An Investigation into the Neuroprotective Effects of Dehydroepiandrosterone

A thesis submitted in fulfilment of
the requirements for the degree of

MASTER OF SCIENCE (PHARMACY)

Faculty of Pharmacy
Rhodes University

By

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December 2005

Knowledge and understanding are life's faithful companions who will never prove untrue to you for knowledge is your crown, and understanding your staff; and when they are with you, you can possess no greater treasure.

I would not exchange the laughter of my heart for the fortunes of the multitudes, nor would I be content with converting my tears, invited by me against self, into calm. It is my fervent hope that my whole life on this planet will ever be tears and laughter.

Kahlil Gibran

For my loved ones

Abstract

Dehydroepiandrosterone, a C-19 steroid, is found endogenously with the highest circulating serum levels. It is converted to important steroids such as the sex hormones oestrogen and testosterone. DHEA has come under the spotlight as a purported "fountain of youth" due to its well-characterised age-related decline. The supplementation of DHEA in both the elderly and those with a pathophysiological deficiency has been shown to be of benefit, particularly with regard to well-being and depression. The role of DHEA in the periphery has not been elucidated beyond its role as a precursor hormone in sex steroid biosynthesis, though it has been established as a neuroactive neurosteroid, capable of exerting neuroprotective effects in the brain.

Since the importance of free radicals in aging and neurodegeneration is well established, investigations were conducted on the ability of DHEA to inhibit free radical generation or scavenge existing free radicals. DHEA was able to significantly inhibit quinolinic acid-induced lipid peroxidation, a measure of membrane damage, over a range of concentrations, although the reduction did not appear to be dose-dependent. This was observed in both *in vitro* and *in vivo* studies. Thus, the ability of a compound to reduce the degree of lipid peroxidation may indicate its value as a neuroprotectant. However, DHEA did not significantly reduce cyanide induced generation of the superoxide free radical, suggesting that DHEA is not an effective free radical scavenger of the superoxide anion and that the reduction in lipid peroxidation does not occur through a scavenging mechanism.

Apoptosis is a physiological process which is necessary for development and homeostasis. However, this form of programmed cell death can be initiated through various mechanisms and too much apoptotic cell death results in deleterious effects in the body. DHEA was shown not to induce apoptosis. Even the lowest concentration of DHEA investigated in this thesis shows a remarkable decrease in the degree of apoptosis caused by intrahippocampal chemical insult by the neurotoxin quinolinic acid. Cresyl violet was used to visualise tissue for histological examination which revealed that DHEA is able to preserve the normal healthy morphology of hippocampal cells which have been exposed to quinolinic acid. Cells maintained their integrity and showed little evidence of swelling associated with necrosis.

Organ culture studies were performed by assessing the impact of DHEA on several pineal metabolites. The study revealed that DHEA exerted an effect on the metabolism of indoleamines in the pineal gland. Melatonin, the chief pineal hormone, did not appear to be affected while the concentrations of N-acetylserotonin, serotonin and methoxytryptamine showed significant alterations. Thus, the neuroprotective mechanism of DHEA does not appear to be mediated by an increase in the presence of melatonin.

The biological importance of metal ions in neurodegeneration is also well established and thus the potential interaction between DHEA and metal ions was considered as a mechanism of action. Spectroscopic and electrochemical analyses were performed to determine whether DHEA is able to interact with metal ions as a ligand. These reveal that DHEA does not form a strong bond with the metals investigated, namely copper (II) and iron (III), but that a weak interaction is evident.

These investigations were conducted in a rodent model, which has neither large amounts of endogenous DHEA, nor the enzymatic infrastructure present in humans. Thus, the theory that DHEA exerts its effects through downstream metabolic products is unlikely. However, these investigations reveal that there is merit in the statement that DHEA itself is a neuroprotective molecule, and confirm that the further investigation of DHEA is an advisable strategy in the war against neurodegeneration and aging.

Acknowledgements

First and foremost, I would like to thank my supervisor Prof Santy Daya for all his assistance with this thesis.

I gratefully acknowledge the assistance provided by Dr Maryna Van De Venter and the Department of Biochemistry at Nelson Mandela Metropolitan University, and for the kind use of their scintillation counter for pineal organ culture studies.

The assistance with the electrochemical studies provided by Dr. Janice Limson and Ronin Fogel of the Department of Biochemistry at Rhodes University is much appreciated.

Thanks to the Rhodes University Electron Microscopy for the use of their equipment for histology and apoptosis studies.

My heartfelt thanks to Sally and Dave Morley for always having a solution, no matter what the problem.

Many thanks to my colleagues at the Neuroscience Research Group for their assistance and support.

I would like to the National Research Foundation and Rhodes University for financial support over the past two years. The finiancial assiatnce from the Henderson Scholarship towards this research is hereby acknowledged. Opnions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to Rhodes University or the donor.

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Abbreviations and Symbols

µl	microlitre
µm	micrometre
3-HAO	3-hydroxyanthranilic acid oxygenase
A	amperes
ACh	acetylcholine
AChE	acetylcholinesterase.
AD	Alzheimer's disease
aHT	N-acetylserotonin
AIDS	acquired immune deficiency syndrome
aMT	melatonin
ANOVA	analysis of variance
ASV	adsorptive stripping voltammetry
BHT	butylated hydroxyl toluene
CPM	counts per minute
Cu(II)	copper 2 or the cupric ion
DHEA	dehydroepiandrosterone
DHEA(S)	dehydroepiandrosterone and dehydroepiandrosterone sulphate
DHEAS	dehydroepiandrosterone sulphate
E	potential
Eqn	equation
Fe(II)	iron 2 or the ferrous ion
Fe(III)	iron 3 or the ferric ion
GCE	glassy carbon electrode
HA	5-hydroxyindole acetic acid
HIOMT	hydroxyindole-O-methyltransferase
HL	5-hydroxytryptophol
HT	serotonin
I	current
IR	infrared

kg	kilogram
L	ligand
M	molar
MA	5-methoxyindole acetic acid
MCR	metabolic clearance rate
MDA	malondialdehyde
MDA	malondialdehyde
mg	milligram
ML	5-methoxytryptophol
ml	millilitre
mM	millimolar
MPP+	1-methyl-4-phenylpyridium
mRNA	ribonucleic acid
NAT	N-acetyltransferase
ND	neurodegenerative disease
NMDA	N-methyl-D-aspartate
nmol	nanomole
PBS	phosphate buffered saline
PUFA	polyunsaturated fatty acid
QA	quinolinic acid
QPRT	quinolate phosphoribosyl transferase
RNS	reactive nitrogen species
ROS	reactive oxygen species
SLE	Systemic Lupus Erythematosus
SOD	superoxide dismutase
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive species
TCA	trichloracetic acid
Tdt	terminal deoxynucleotidyl transferase
TLC	thin layer chromatography
UV	ultra violet

V	volts
VIS	visible
w/v	weight in volume

Literature Review

1.1 Neuroscience

The brain has not always been recognised as a vital organ. In fact, Egyptians of the middle Kingdom (c. 2040-1786 B.C.) would scrape the brain out through the nostrils of corpses prior to mummification and dispose of the tissue, in sharp contrast to the preservation of the abdominal organs. Over a thousand years later the situation had improved somewhat when Plato (429-347 B.C.) proposed that the head controlled reason and perception – although he also suggested that the heart and lungs were responsible for the "Noble Passions" such as courage and pride. Later still, Aristotle (384-322 B.C.) is credited with establishing the heart as the organ where sensations occurred, as touching the brain does not result in sensation being experienced (Gellatly & Zarate, 1998). And as recently as the 18th Century, the belief was widespread that the contours of the skull were linked to 32 character traits. This idea was proposed by the Viennese doctor, Franz Gall who named the discipline phrenology (Greenfield, 1997).

Fortunately we are currently in a more enlightened era – the nineties were "The Decade of the Brain" – though doubtless the neuroscientists of the future will also be citing our mistakes. Neuroscience has come a long way and numerous journals and societies are now dedicated to this specialisation.

1.2 Neuroanatomy

High school biology teaches us that the basic unit of the brain is the neuron, as depicted below in Fig 1.1). The human brain consists of a huge number of intercommunicating neurons, in the order of 10^{11}.

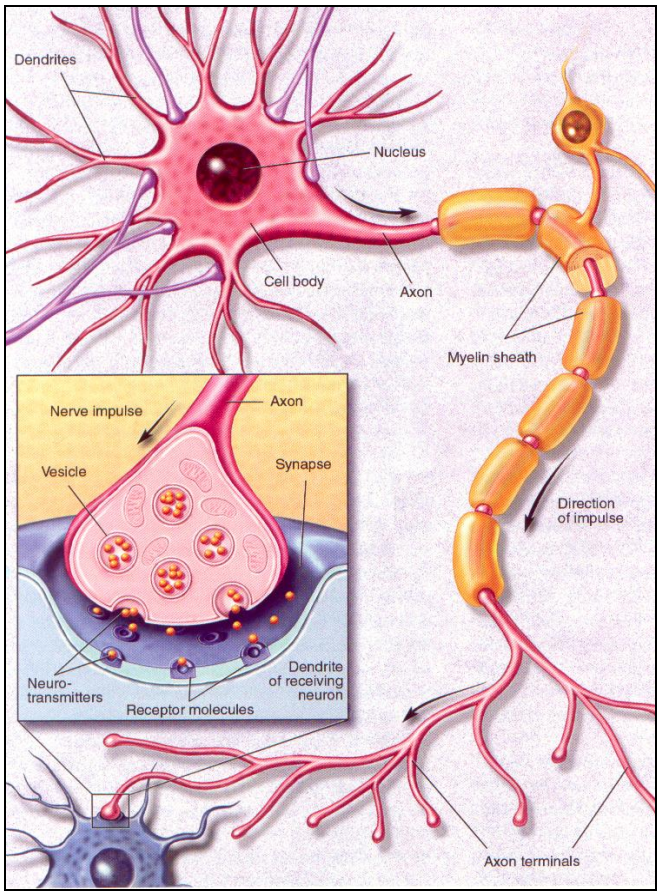


Fig 1.1 The diagram shows a neuron, consisting of a central mass of cytoplasm with a cell membrane (soma) as well as numerous neuritic extensions. The axon conducts information away from the cell body, while multiple dendrites conduct information to the cell body (Standring, 2005; Carey, 2005).

Neurons are polarised and conduct information in the form of action potentials from the patterns of depolarisation and repolarisation of the membrane potential. The potential when resting is approximately -70mV with respect to the outside of the neuron. Communication between neurons may exist in multiple configurations with the axon terminal of one neuron forming a synaptic contact with dendrites, axons, cell bodies or non-neural tissue such as muscle cells (Standring, 2005). In

addition to the neurons, glia are present in the brain, although the current opinion is that they serve in a supportive function. The most abundant glial cells are astrocytes, followed by myelinating glia and other non-neuronal cells cells such as ependymal cells and microglia (Bear *et al*, 2001)

The nervous system may be categorised as the central (CNS) or peripheral nervous system (PNS), although the former, consisting of the brain and spinal cord is of the most relevance to this thesis. The brain is divided into major regions, based on ontogenetic growth and phylogenetic principles. The rhombencephalon is proximal to the spinal cord and comprises of the medulla oblongata, the pons and the cerebellum or "little brain". The medulla is caudal to the rest of these areas and is continuous with the brain stem below the foramen magnum (Standring, 2005). The pons and medulla are responsible for several autonomic and homeostatic functions including respiration and digestion. All the sensory and motor neurons which pass to and from higher brain regions pass through the hindbrain, making conduction of information one of the most important functions of the medulla and pons (Campbell, 1990).

The next major division is the prosencephalon consisting of the lower diencephalon and upper telencephalon. The former contains the hypothalamus, hypothalamic, subthalamus and epithalamus, whereas the telencephalon consists of the two cerebral hemispheres, which are the largest portion of the brain (Standring, 2005). These hemispheres are separated by the saggital fissure (Morgan & Butler, 1993), although the right and left cerebral hemispheres are linked by various commissures, the largest and most important of which is the corpus callosum, allowing for communication between the hemispheres.

The surface of the brain is highly convoluted and these convolutions are more highly developed in higher mammals. Sulci, or "fissures", and gyri ("ridges") give rise to these convolutions and allow for a greater surface area of the brain within the cranium. When a saggital section is taken through the brain, the grey and white matter are clearly visible. Grey matter forms the cerebral cortex and below this lies the white matter containing nerve fibres which communicate with the cerebral cortex. The basal ganglia are comprised of grey matter, but are embedded in the white matter. Four ventricles exist within the brain and are filled with cerebrospinal fluid which is secreted by the choroids plexus (Standring, 2005).

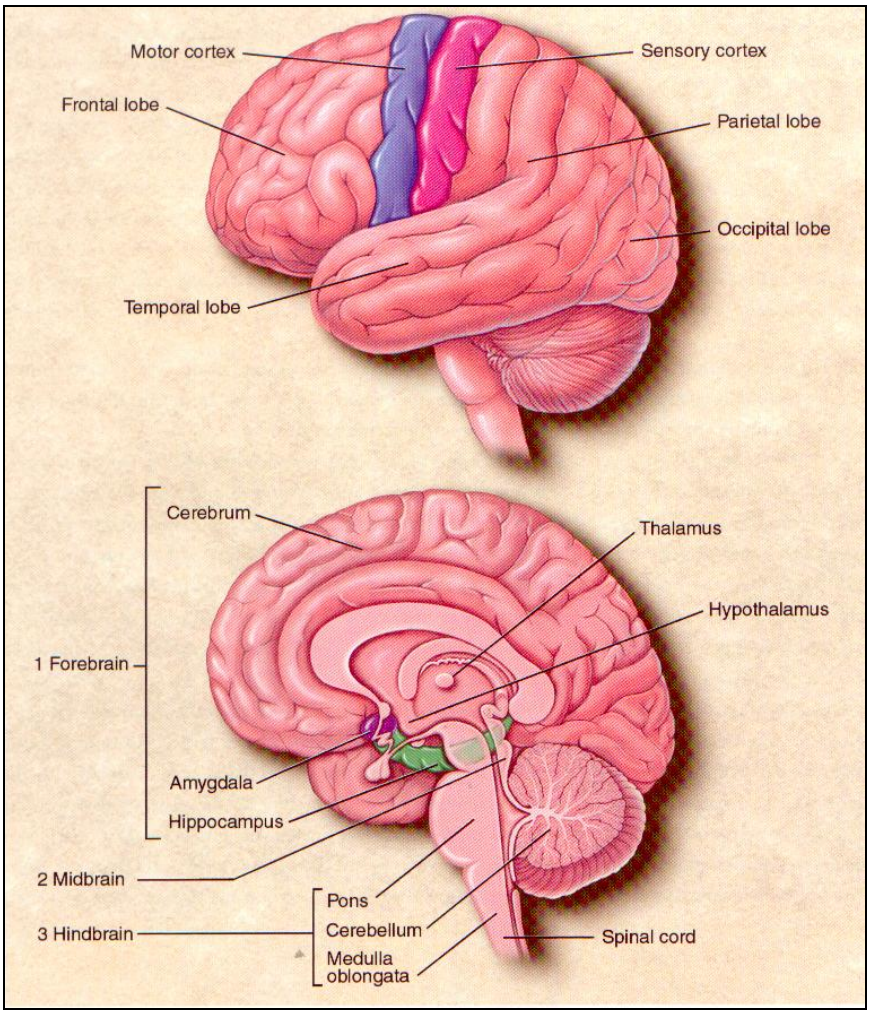


Fig 1.2 Diagrammatic representation of the main features of the brain mentioned above (Carey, 2005).

1.2.1 The Hippocampus

The term hippocampus is derived from the Greek *hippos* meaning horse and *kampos*, referring to a sea-monster (www.oed.com). This brain structure of the limbic system, found on both lateral ventricles, is named for its resemblance to the seahorse. This similarity may be noted below in Fig 1.3.

Julius Caeser Arantius designated this structure the hippocampus in the late 16th century and the term Ammon's horn or *Cornu Ammonis* has been favoured through modern history. The term finds its origins in Egyptian mythology where the god Amun was represented as a head with ram-like horns

(Pearce, 2001). The regions of the hippocampus that are designated CA1, CA2 and CA3 derive their names from this Latin term.

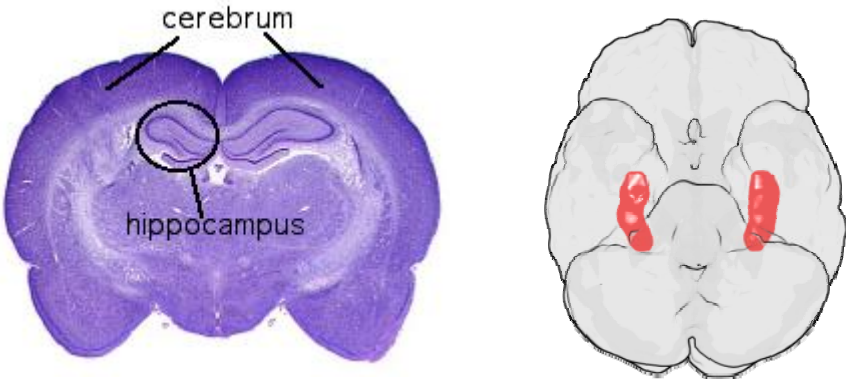


Fig 1.3 The figure on the left shows the location of the hippocampus in the rat brain in the transverse plane (http://www.bio.davidson.edu/courses/genomics/method/Brainparts.html), while the diagram on the right shows the location of the hippocampus in the temporal lobe of the human brain from a ventral view, where the hippocampus (red) is within the transparent brain (grey).

1.2.1.1 The Structure and Function of the Hippocampus

The hippocampus is situated medially to the lateral ventricle in the medial temporal lobe as demonstrated above in Fig 1.3. The glutamatergic system is the chief pathway involved in the functioning of the hippocampus which is intrinsic to learning and memory (Bear *et al*, 2001) and the hippocampus, along with the parahippocampal region and areas of the prefrontal cortex is responsible for declarative or cognitive memory (Carey, 2005). It is required for the formation of new memories, and those with damaged hippocampi are affected in this regard. The website Newscientist.com published a news piece on 12 March 2003 about a prosthetic hippocampus, consisting of a silicon chip which would allow those with damaged hippocampi to form new memory.

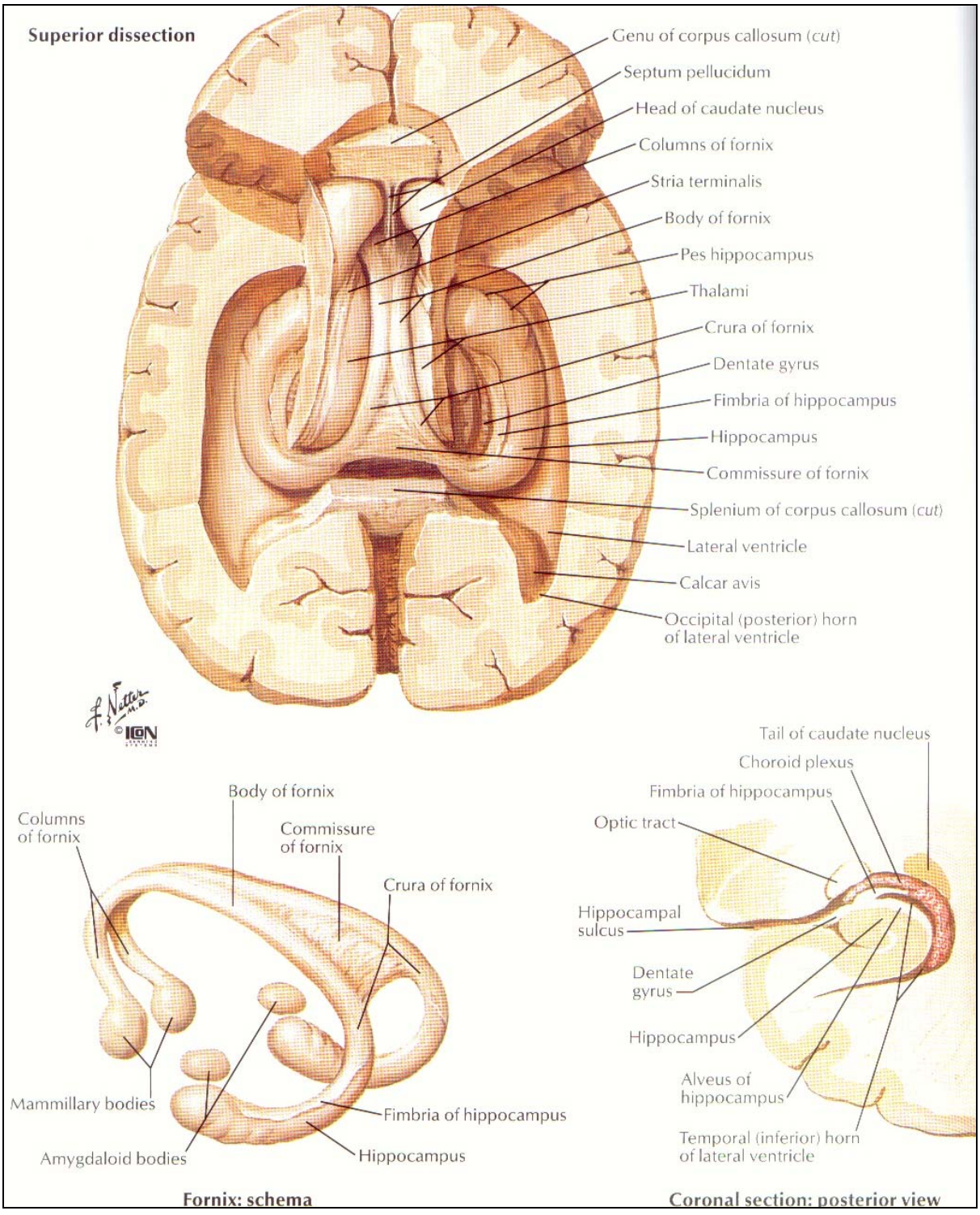


Fig 1.4 The diagram above clearly shows the location and anatomy of the hippocampus (Hansen, 2003).

The hippocampus consists of neurons of the dentate gyrus (DG) and Ammon's Horn which are folded together in thin sheets. The hippocampus receives input from the entorhinal cortex *via* the perforant pathway, which consists of a bundle of axons. These axons communicate with the DG through synaptic connections. The DG neurons produce mossy fibres which in turn communicate with neurons of the CA3 region. Two main axonal branches of this region communicate with the fornix and the Schaffer collateral, connected to the CA1 region (Bear *et al*, 2001).

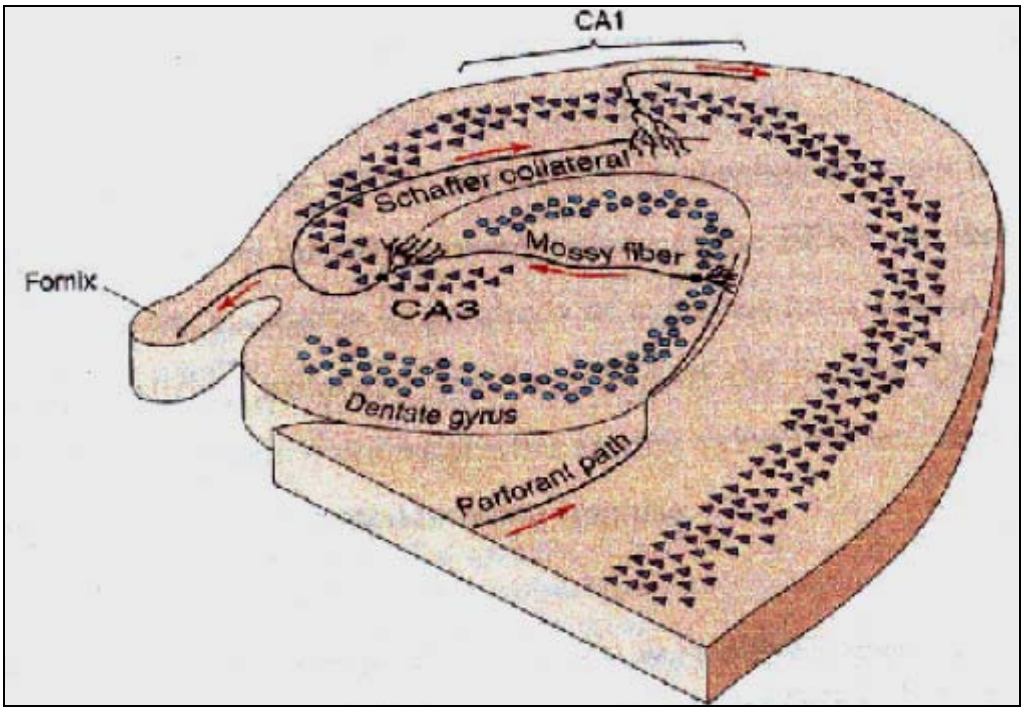


Fig 1.5 The illustration shows the microcircuits of the hippocampus as described above (Bear *et al*, 2001).

1.2.2 The Pineal Gland

The pineal gland, identified initially by Hierophilos (325-280 BC) has historically been linked with spirituality, by the Ancient Greeks as a link to the Realms of Thought and most famously as Descartes' "seat of the soul". It is colloquially referred to as the "third eye" and many believe that an "awakening of the third eye" may lead to spiritual enlightenment and a fuller life. Even more interesting than its significance to mysticism and spirituality is its somewhat more demonstrable significance to neuroscience.

1.2.2.1 The Structure and Function of the Pineal Gland

The gland is located on the dorsal surface of the hypothalamus, between the cerebral hemispheres in the human (Ebadi, 1993). While in the rat brain, its location is more dorsal. It is easily visible on the surface of the brain, as shown in Fig 1.6.

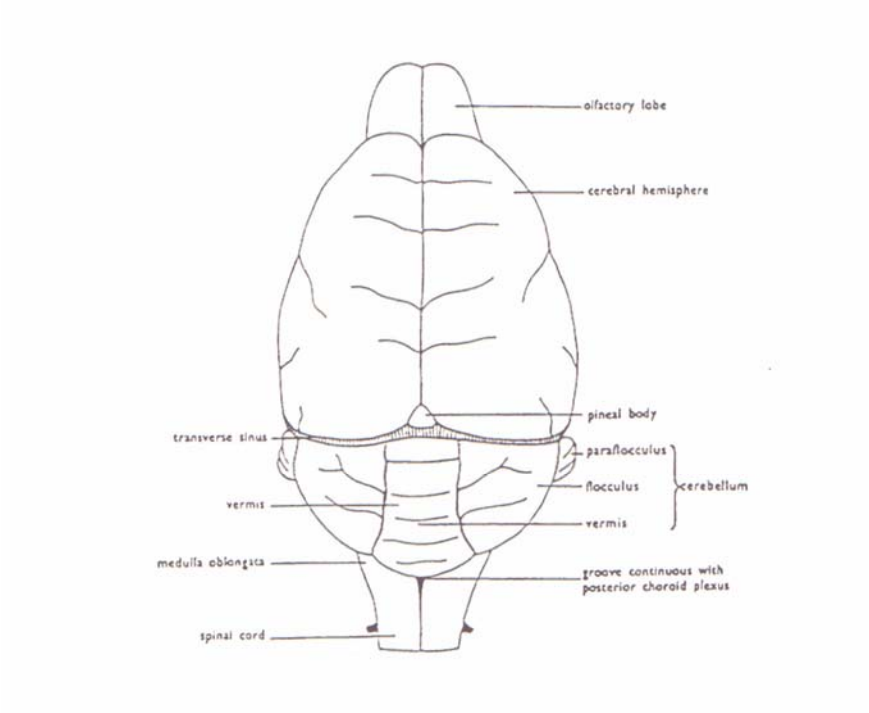


Fig 1.6 The pineal gland is visible anterior to the transverse sinus (Rowett, 1962).

It is a highly vascularised tissue consisting of pinealocytes which predominate, and neuroglia. These pinealocytes produce both melatonin and peptides e.g. arginine-vasotocin (Brzezinski, 1997). The pineal gland is innervated by the sympathetic nervous system and is responsive to light intensity through the control of the supra-chiasmatic nucleus, and produces melatonin (Ebadi *et al*, 1986).

1.2.2.2 Metabolism of Indoleamines by the Pineal Gland

Pineal indole metabolism is responsible for the production of the indoleamine melatonin from tryptophan in the pinealocytes (Reiter *et al*, 2000) and the synthesis of melatonin exhibits a circadian rhythm that is well documented (Reiter, 1987; Lam *et al*, 2004). The production of melatonin declines with age when susceptibility to immune dysfunction, cancer, infectious disease and oxidative damage increases (Zhang *et al*, 1999).

Further description of the metabolism of the pineal gland, as well as a diagram detailing its three main metabolic pathways and enzymes involved in these syntheses is included in *Chapter 6: Pineal Organ Culture Studies.*

1.3 Neurotoxins

Neurotoxins are substances that are poisonous to nervous tissue. Some substances are endogenous to organisms, yet when concentrations are raised, these become neurotoxic. An example of this is quinolinic acid, discussed below. Chemicals can be used as neurotoxins to mimic destruction in certain pathological conditions such as the use of MPTP in a model for Parkinson's Disease (Freyaldenhoven *et al*, 1997), and kainic acid (Leite *et al*, 2002) and pentylenetetrazole (Hansen *et al*, 2004) to induce seizures or epileptogenesis.

1.3.1 Quinolinic acid as a neurotoxin

Quinolinic acid (QA or 2,3-pyridine dicarbolic acid) is an amino acid which occurs endogenously in the human brain. This amino acid is structurally related to L-glutamate and L-aspartate and is a metabolite in the tryptophan-kynurenine pathway (Lapin, 1978), as shown below in Fig 1.7.

QA is produced by activated monocytes and macrophages *in vitro* (Saito *et al,* 1993) and human microglia have been shown to convert the precursor L-tryptophan to QA (Heyes *et al*, 1996).
QA is a potent activator of the N-methyl-D-aspartate (NMDA) group of glutamate receptors (Stone and Perkins, 1981; Stone, 2001a), thereby exerting its neurotoxic properties. The authors showed it to be approximately 25% as active as NMDA itself. Unlike its structural analogues L-glutamate and L-aspartate, a high affinity, rapid uptake system for QA is not present in the brain (Foster *et al*, 1984).

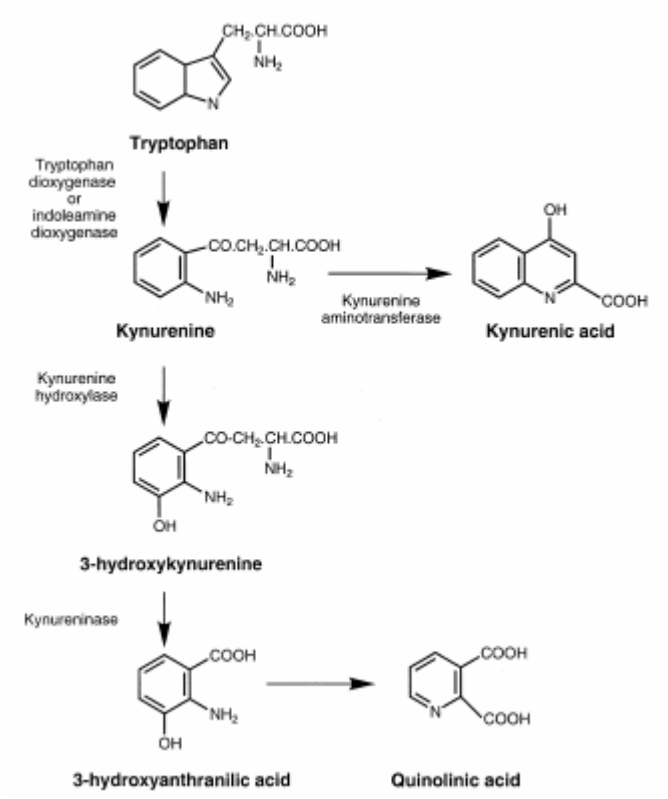


Fig 1.7 The diagram illustrates the metabolic pathway of quinolinic acid (Stone, 2001).

The enzyme 3-hydroxyanthranilic acid oxygenase (3-HAO) is responsible for the synthesis of QA from its precursor, 3-hydroxyanthranilic acid, and the reaction velocity of this enzyme has been shown to be 80 times faster than that of quinolate phosphoribosyl transferase (QPRT), the enzyme which metabolises QA to its degradation products (Foster *et al*, 1986). 3-HAO has been found in areas of the brain such as the hippocampus which have little detectable QPRT so the introduction of increased levels of QA may lead to excitotoxic damage and a cognitive decline and memory loss resembling Alzheimer's disease (Stone, 1993).

The combination of these factors explains the potency of QA, an endogenous substance, as a neurotoxin since it is not adequately removed from the synaptic cleft and can thus continue to stimulate NMDA receptors.

Although QA is found endogenously, its physiological role is still unclear. However, researchers have determined that QA is found in increased concentrations in various pathological conditions, including

convulsions (Lapin, 1981), Huntington's Disease (Schwarcz *et al*, 1986), hepatic encephalopathy (Moroni *et al*, 1986) and AIDS-related neurological disorders (Brown, 1990). It is implicated in many neurodegenerative diseases (Beal *et al,* 1986).

QA exposure results in excitotoxic damage characterised by raised cytosolic Ca^{2+} concentration, depletion of ATP and gamma-aminobutyric acid GABA, as well as oxidative cell death (Santamaría *et al*, 2003). The ability of QA to induce seizures and direct neural death when injected intracerebroventricularly has been demonstrated (Lapin, 1978; Schmidt *et al*, 2000; Lara *et al*, 2001). Schauwecker (2002) reported that 120nmol QA administered intrahippocampally produces Stage 5 seizures in 75% of mice, including postural imbalance followed by clonic or tonic-clonic convulsions.

Rios & Santamaria demonstrated in 1991 that QA induced lipid peroxidation and that it has been suggested that the free radical NO may be involved in the neurotoxicity of QA (Santamaria *et al*, 1997). The authors base their suggestion on the ability of nitroarginine to inhibit and L-arginine to potentiate the lipid peroxidation caused by QA. Southgate (1998) provided evidence that melatonin, a well-established free radical scavenger, was able to protect against QA induced damage. Additionally, Fe^{2+} ions are released during neuronal damage for intracellular stores and the activity of 3-HAO may be reduced by the presence of Fe^{2+} ions (Stone, 1993).

1.3.2 Cyanide as a Neurotoxin

Cyanide has been known to be poisonous and is very rapidly lethal due to the interruption of the mitochondrial electron transport chain. Death can occur within 5 minutes, making it the suicide method of choice of Alan Turing, often referred to as the father of computer science, as well as Adolf Hitler and Eva Braun. In the early 1980's there was widespread panic in the USA after containers of Tylenol® were laced with cyanide.

Enzymes are present in the human body to cope with trace amounts of cyanide and the normal blood value for cyanide is below 3.8 µmol/L (Beers & Berkow, 1999). It is a respiratory poison and cyanide poisoning normally results in fatality due to the vulnerability of nerve cells of the respiratory centre to hypoxia (Greer & Jo, 1995).

Cyanide has been demonstrated, through various biochemical, morphological and physiological means to be a potent and selective neurotoxin whose toxicity is mediated through histotoxic hypoxia,

consequent to mitochondrial dysfunction (Bhattacharya & Lakshmana Rao, 2001). Cyanide inhibits Complex IV (cytochrome c oxidase) which is the terminal electron acceptor in the mitochondrial electron transport chain (Isom & Way, 1984). This cyanide-induced dysfunction results in a cascade of effects consisting of failure of ionic homeostasis, acidosis, elevated Ca^{2+} levels and lipid peroxidation, leading to activation of proteases, lipases and xanthine oxidases (Maduh, 1989). The elevation in brain calcium (Johnson *et al*, 1986) and increase in free cytosolic calcium leads to increased oxidative stress and excitotoxcity.

Cyanide exposure results in oxidative stress and activation of endonucleases in oligonucleosomal cleavage of DNA (Trump & Berezesky, 1995). Subsequent to oxidative stress, widespread DNA fragmentation leading to an apoptotic type of cell death has been documented in cyanide poisoning (Mills *et al*, 1996; Mills *et al*, 1999), with Mills and colleagues (1996) reporting an apoptotic effect of cyanide in terminally differentiated PC12 cells. Cyanide also causes surface blebbing and cytoarchitectural defects of neuronal cells as a consequence of Ca^{2+}-activated phospholipases and proteases (Nicotera *et al.*, 1989).

1.4 Excitotoxicity

Excitotoxicity is the ability of excitatory amino acids (EAA) to excessively stimulate post-synaptic receptors, thereby causing neuronal degeneration (Olney, 1995). Glutamate, the chief excitatory neurotransmitter in the mammalian brain, is important in various physiological and pathological events, and is an example of such an EAA. Glutamatergic neurotransmission occurs through ionotropic (ligand-gated ion channels) and metabotropic (G protein-coupled) receptors (Ozawa *et al*, 1998). The NMDA ionotropic glutamate receptor appears to be particularly vital for normal brain function, whereas excessive stimulation of the glutamatergic system may be neurotoxic (Izquierdo & Medina, 1997; Ozawa *et al*, 1998). This overstimulation occurs when the synaptic cleft concentration of glutamate is increased.

EAAs may produce neurotoxicity in one of two principle manners which may be distinguished by their time course and ionic dependence.

Acute excitotoxicity may be identified by the rapid influx of sodium ions into the neuron due to excessive depolarisation, accompanied by passive entry of water and chloride ions due to osmotic pressure. Thus the neuron swells (Tilson & Mundy, 1995).

Delayed excitotoxicity is NMDA receptor mediated and involves the influx of calcium ions (Ca^{2+}). This raises the intracellular concentration of Ca^{2+} which in turn triggers several neurotoxic enzymatic and metabolic processes, including the activation of calpain-1, a protease which degrades the cytoskeleton (Seubert et al, 1988). The calcium ion may form Ca^{2+}-calmodulin, which is well established as an enzyme modulator. It may result in mitochondrial dysfunction or activate lipases (Scatton, 1994).

The phospholipases which catabolise cell membranes are activated by Ca^{2+}, resulting in the destruction of Ca^{2+} stores within the cell and exacerbating an already injurious situation. Arachidonic acid is liberated during this process, allowing for the synthesis of prostaglandins. These biological molecules are important in the inflammatory response and inflammatory cells produce reactive oxygen and nitrogen species (ROS and RNS, respectively) as a mechanism for opsonised targets (Akiyama *et al*, 2000).

It has been established that increasing the expression of neuronal nitric oxide synthase (NOS) in culture results in increased glutamate neurotoxicity. Neuronally originated nitric oxide is therefore important in behavioural function and as an excitotoxic trigger (Dawson *et al*, 1993).

In the discussion of free radicals earlier in this chapter, it was noted that excess nitric oxide generation in the neuron may result in the production of the peroxynitrite radical if the superoxide anion is present. Thus excitotoxins increase free radical production, resulting in excess release of glutamate extracellularly. The entry of Ca^{2+} during delayed excitoitoxicity activates NOS and the cycle continues (Blaylock, 1999). The mediators result in activation of the caspase cascade, and then in apoptotic cell death (Cassarino & Bennett, 1999).

1.5 Free Radicals and Oxidative Stress

Free radicals are any moieties, such as atoms or molecules, which possess unpaired electrons in their outermost orbit. Due to this, free radicals are largely unstable and highly reactive (McMurry, 2000). They are well established as being necessary in many biochemical processes such as signal transduction and gene description (Zheng & Storz, 2000), but their effects may be deleterious due to their oxidant nature which harms cells and may result in cell death (McCord, 2000).

Atmospheric oxygen exists as O_2 and this molecule is a very powerful oxidising agent due to its ability to easily accept electrons from other molecules. Any free radical including oxygen is referred to as a reactive oxygen species or ROS.

The aging process is accompanied by an increase free radical production due to normal processes and the presence of ultraviolet light. The introduction of toxins also results in increased oxidative insult (Reiter, 1995). The brain has a high metabolic activity and consumes a major proportion of total body oxygen (Halliwell, 1992), as much as 20%, even though it masses only 2% of body weight (Juurlink & Paterson, 1998). The brain also has a high lipid content which makes it readily susceptible to lipid peroxidation after free radical attack, as well as a high iron content required for Fenton initiated free radical generation (Reiter, 1998). Not all oxygen in cells is used for the production of ATP and can therefore be converted to reactive oxygen species (ROS).

1.5.1 Types of Free Radicals

1.5.1.1 The Superoxide Radical

When molecular oxygen is reduced through the acceptance of an electron, the superoxide anion ($O_2^{\bullet -}$) is created. This moiety exists in aqueous environments in equilibrium with its conjugate acid, the hydroperoxyl radical, and both have been shown to participate in various biotransformations, including the oxidation of α-tocopherol to 8α-tocopherone (Nishikimi *et al*, 1980). The superoxide anion also mediates the reduction of cytochrome c by xanthine and xanthine oxidase (McCord & Freidovitch, 1968). This reaction is used in the assay for both the superoxide anion and superoxide dismutase, which are enzymes which have evolved a surface charge arrangement to make specific use of the superoxide anion as substrate (Benovic *et al*, 1983). Activated cells such as macrophages produce superoxide to aid in the phagocytation and subsequent destruction of bacterial cells (Colton & Gilbert, 1987), which explains how chronic inflammation can lead to further damage mediated by free radicals.

The superoxide free radical is implicated in a broad spectrum of diseases due to its toxicity which results from its ability to inhibit various enzymes and metabolic pathways. It has varied effects on some of the major classes of biological molecules (McCord, 2000).

This radical alters DNA, directly or indirectly as demonstrated by increased rates of mutagenesis in *E. coli* with SOD impairment (Touti & Farr, 1990).

The superoxide radical may be involved in the initiation and termination steps of lipid peroxidation (as shown in Fig. 1.7 below). Reperfusion after ischaemia results in increased production of SOD and is associated with increased lipid peroxidation (Nelson *et al*, 1994).

The superoxide radical generated intracellularly is readily converted to hydrogen peroxide through the acceptance of a single electron and two protons.

1.5.1.2 The Hydroperoxyl Radical

As mentioned previously, this is the conjugate acid of the superoxide radical, created by its protonation. The pK_a of the hyroperoxyl radical is relatively acid, so at physiological pH little is present (Bielski & Allen, 1977). However, while physiological pH is accepted as approximately 7.4, in isolated areas of the body, the pH may drop, increasing the relative amounts of the hydroxyproxyl radical. An example of such a location is in close proximity to membranes.

The hydroperoxyl radical is less polar than its conjugate base, so is more easily able to cross lipophilic membranes (Halliwell & Gutteridge, 1986), as well as being more reactive than the superoxide radical and may attack lipids directly, converting linolenic, linoleic and arachidonic acids to peroxides (Bielski *et al*, 1983).

1.5.1.3 The Hydroxyl Radical

Reiter (1995) refers to the hydroxyl radical as "the radical's radical" since it may react with any molecule it encounters at diffusion rates. It is arguably one of the most highly oxidising entities and is a "molecule destroyer".

Hydroxyl radicals may be generated from superoxide and hydrogen peroxide via two main routes: the Haber-Weiss reaction or the Fenton reaction.

In the Haber-Weiss reaction, superoxide interacts with hydrogen peroxide to produce the hydroxyl radical as well as a molecule of water and a hydroxyl group as demonstrated in Equation 1.

In the Fenton reaction (Fenton, 1894), ferric ions interact with superoxide anions, producing ferrous ions, which in turn interact with hydrogen peroxide to produce the hydroxyl radical as demonstrated in Equations 2 and 3.

$O_2^{\bullet -} + H_20_2 \rightarrow OH^- + H_20 + OH^{\bullet}$ Eqn 1

$Fe^{3+} + O_2^{\bullet -} \rightarrow O_2 + Fe^{2+}$ Eqn 2

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$ Eqn 3

It is widely recognised that the hydroxyl radical is so highly reactive that it will interact with any molecule within a few Angstroms of its site of production, and its half life at physiological temperatures may be measured in nanoseconds. These molecules may be DNA, membrane lipids or carbohydrates (Dawson & Dawson, 1996, Reiter *et* al, 1995) and no enzyme system uses the hydroxyl radical as a substrate (Bird & Iversen, 1974).

1.5.1.4 Nitric Oxide and the Peroxynitrate Radical

Nitric oxide (NO) is an important physiological messenger whose roles include modulating the pyloric sphincter (Yun *et al*, 1996) and vasodilation (Holsher, 1997). It is released most commonly by vascular endothelial cells and phagocytes (Moncada *et al,* 1991) and one of the properties which makes it an effective messenger is its short half-life, reported to be approximately six seconds. This is explained by the presence of the superoxide radical which rapidly converts it to the peroxynitrate radical (Beckman *et al,* 1993).

The peroxynitrate radical is highly reactive and may be able to diffuse across several cell diameters, damaging cells through the oxidation of lipids, proteins and DNA (Beal, 1997). The inflammatory hypothesis of AD suggests that peroxynitrite production is a factor worsening neuronal degeneration (Torreilles *et* al, 1999)

1.5.2 Lipid Peroxidation

This reaction is defined as the oxidative degradation of polyunsaturated fatty acids or PUFAs, found in cell membranes. Lipid peroxidation is a radical reaction and, as such, proceeds through three clearly defined steps: initiation, propagation and termination (McMurry, 2000).

This degradative process can occur in the presence of any species with enough activity to abstract a hydrogen atom from an unsaturated carbon double bond such as the methylene group present in large quantities in lipids. Examples of these highly reactive initiator moieties include hydroxyl radicals and iron-oxygen complexes. Cell damage has been shown to produce hydroxyl radicals. Abstraction is assisted by the presence of the carbon double bond, since the adjacent carbon-hydrogen bond is weakened. The abstraction results in an unpaired electron and the formation of a carbon radical in the initiation step. The carbon radical is fairly unstable so the lipid undergoes molecular rearrangement to form a more stable conjugated diene. This diene combines with oxygen under appropriate conditions to form the peroxyl radical, $ROO^{\cdot}$.

The peroxyl radical then abstracts hydrogen from another lipid molecule during the propagation step. Another carbon radical is formed and it may react to form another peroxyl radical, continuing the chain reaction. The length of this propagation chain is affected by the lipid:protein ratio in the membrane, the fatty acid composition, as well as the oxygen concentration and the presence of chain terminating anti-oxidant moieties. These molecules provide easily donatable hydrogens and are abstracted by the peroxyl radicals in favour of lipid molecules during the termination step (Halliwell & Gutteridge, 1990). Both peroxyl radicals and lipid peroxides attack macromolecules such as intramembranous protein receptors or enzymes (Reiter, 1995) which can have a deleterious effect on cell function.

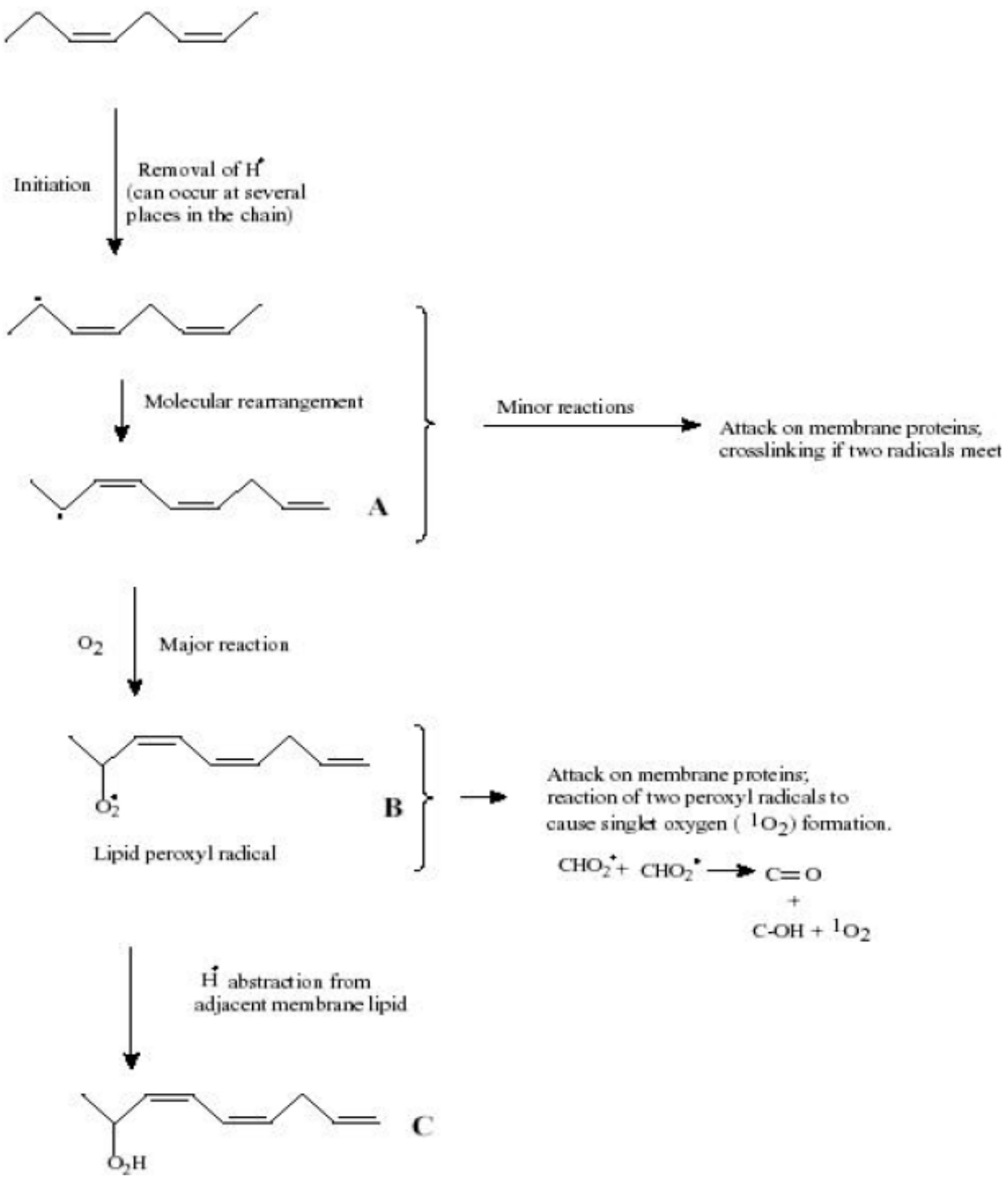


Fig 1.8 Diagrammatic representation of the mechanism of lipid peroxidation (Gutteridge & Halliwell, 1990).

1.6 Cell Death

Cell death may be broadly classified into two morphologically and biochemically distinct types, namely necrosis and apoptosis. Necrotic cell death is pathological whereas apoptosis is normal physiological process (Wylie *et al*, 1998). Whether cell death proceeds via apoptosis or necrosis is dependent on the type and duration of oxidative insult.

1.6.1 Distinguishing between Apoptosis and Necrosis

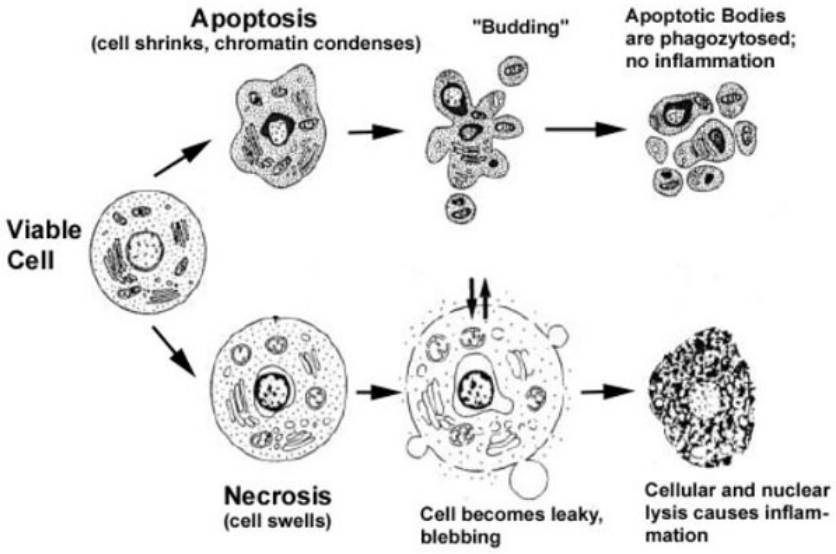


Fig 1.9 The distinct morphological changes which occur during the processes of apoptosis and necrosis are easily visible (www.cell-death.de/aporeview).

1.6.2 Necrosis

Cell death via necrosis occurs as a result of various pathological stimuli and is a passive process – membrane integrity is disturbed, cellular swelling and subsequently lysis occurs. Necrotic cell death occurs very rapidly, in contrast to the delayed onset of apoptotic cell death. Necrosis affects neighbouring cells and results in a significant inflammatory response (Van Furth & Zwet, 1988), as well as a loss of regulation of ion homeostasis. It may be evoked by non-physiological stimuli such as lytic viruses, ischaemia and metabolic processes. DNA is digested randomly (Gschwind & Huber, 1997) and no vesicles are formed, so lysis is total, liberating cell contents. The organelles swell and disintegrate (Wylie *et al*, 1998). A primary effect of cell necrosis is lipid peroxidation of cell

membranes. This results in damage to surrounding tissues by destroying lipid membranes and the formation and release of toxic products.

1.6.3 Apoptosis

Lockshin and Williams (1964) suggested that programmed cell death follows a sequence of controlled steps leading to locally and temporally defined self-destruction. The term apoptosis was first used by Kerr *et al* (1972) and is derived from Greek, meaning a falling or dropping off, in reference to leaves falling off trees, or petals falling off flowers. Technically, apoptosis refers only to the morphological changes while programmed cell death is more appropriately used to describe the fundamental processes.

The terms programmed cell death and apoptosis are frequently used interchangeably, but Schwartz *et al* (1993) maintain this is inaccurate as the authors demonstrated that not all cell death displays the characteristics of apoptotic cell death. Another form of cell death (PCD2) exists in addition to apoptosis (PCD1) and is termed autophagy (Petersen *et al,* 2001). Characteristics include membrane wrinkling, nuclear pyknosis (condensation) and the retention of high molecular weight genomic DNA (Bursch *et al,* 2000).

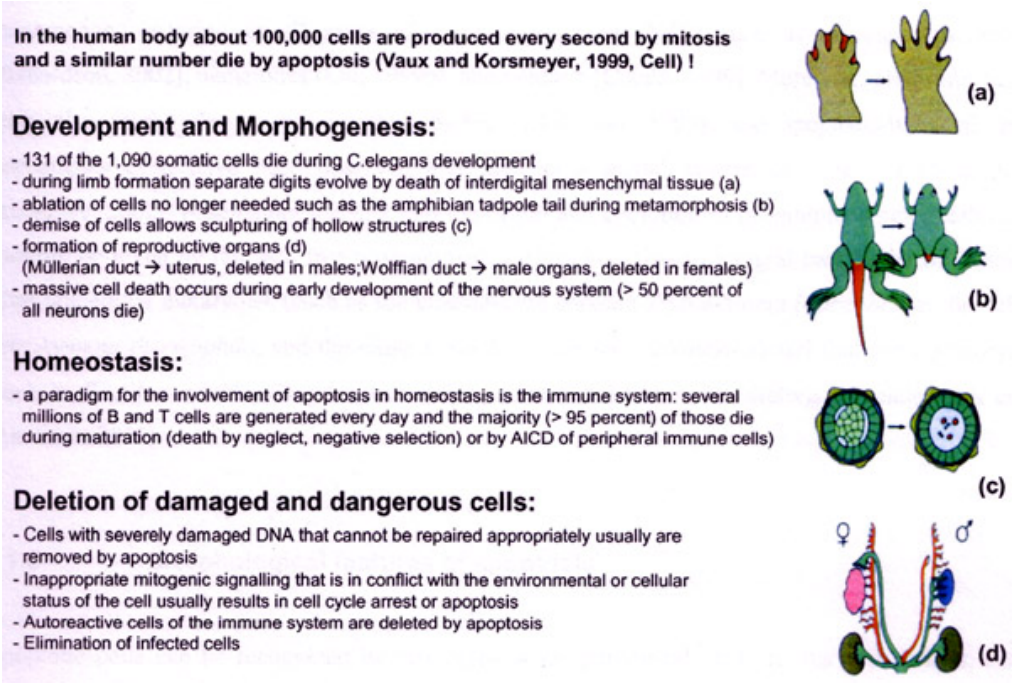


Fig 1.10 The diagram illustrates the effect of programmed cell death on various organisms and demonstrates the necessity of this process (www.celldeath.de/aporeview).

Cell death is not necessarily a negative occurrence. It is in fact vital for development (Clarke, 1990; Johnson & Deckwerth, 1993; Batistatou & Greene, 1993). For instance, the formation of the digits occurs through extensive cell death of the mesenchymal tissue originally present interdigitally. It has also been noted that massive cell death occurs during early development of the nervous system, such as at the edge of the neural plate and within the optic stalk (Kuan *et al,* 2000). Other instances where cell death is necessary for development are shown in Fig 1.10 above.

Hamburger and Levi-Montalcini (1949) showed that removal of limb buds in chick embryos resulted in histiogenetic degeneration of the corresponding spinal ganglia. Kuan *et al* (2000) conclude that cell death modifies the initial progenitor pool, thereby modifying the developing nervous system.

Apoptosis is the most common form of eukaryotic cell death (Wylie *et al*, 1998) and is a homeostatic process, since a balance must exist between the constant formation of new cells via mitosis and the death of old cells. The process requires the *de novo* expression of genes and activation of these genes (Schwartz, 1991) and is thus an active process.

However, the inappropriate regulation of apoptosis may play an important role in several pathological conditions including stroke, cancer, AIDS and neurodegenerative disorders (Delhalle *et al*, 2003).

1.6.4 Features of Apoptosis

Apoptotic cell death results in characteristic biochemical and morphological changes as well as chromosomal DNA organisation (Cohen, 1993). The cell shrinks and is unable to maintain contact with neighbouring cells. The chromatin in the cells condenses. Budding, or blebbing, occurs resulting in the formation of apoptotic bodies, the occurrence of which is a primary diagnostic feature of apoptosis (Kerr *et al*, 1972). These bodies contain cytosol, the condensed chromatin, as well as organelles. These are engulfed by macrophages so no cellular contents are released and no inflammatory response results. These phagocytic cells secrete cytokines such as IL-10 and TGF-β which in turn inhibit the inflammatory response. This is represented graphically in Fig 1.9 above.

The fragmented DNA results in a characteristic ladder pattern in agarose gels (Wyllie, 1980). The morphological changes mentioned above are initiated by activating members of the protease family called cysteine aspartate-specific proteases or caspases (Delhalle *et al*, 2003).

Apoptosis may occur through one of two pathways, referred to as the extrinsic and intrinsic pathways. When apoptosis occurs via the extrinsic pathway or “death receptor” the process is

triggered by activation of the tumour necrosis factor (TNF) superfamily which then recruits several procaspases. The intrinsic pathway is the mitochondrial pathway and occurs due to mitochondrial release of factors which also activate procaspase. This activation of procaspases leads to either activation or inhibition of target proteins which ultimately leads to apoptosis (Dehalle *et al*, 2003).

Many conditions can result in cell death *via* apoptosis, and pharmacological agents such as anti-oncogenic drugs have their effect through this form of programmed cell death. Of particular relevance is the contribution of apoptosis to several neurodegenerative disorders, including AD (Wylie *et al*, 1998).

1.7 Neuroprotection and Neuroprotective Strategies: Defence against Free Radicals

One of the most documented strategies for the defence against free radicals is the use of antioxidants. These molecules act by attenuating the damage caused by oxidative stress, usually by reacting with intermediary peroxide molecules to inhibit lipid peroxidation. However it must be borne in mind that lipid peroxidation is not the only consequence of oxidative insult (Halliwell, 1992).

Complex endogenous antioxidant defence mechanisms which scavenge ROS (Halliwell & Gutteridge, 1989; Cheeseman & Slater, 1993) include relative small molecules such as tocopherols, ascorbic acid glutathione and melatonin, as well as antioxidant enzyme systems such as superoxide dismutases, glutathione peroxidases, glutathione reductase and catalases (Frei *et al,* 1989; Burton & Ingold, 1989; Behl *et al,* 1997; Murphy *et al*, 1989; Tan *et al*, 1993). These are necessary in normal biological function since free radicals play an integral role in many metabolic pathways and messengering systems as mentioned previously. Steroids have been shown to have neuroprotective properties by acting as antioxidants and inhibiting lipid peroxidation (Behl *et al*, 1997), but this is discussed in more detail later in this chapter.

Vitmain E (alpha-tocopherol) participates in the termination step of lipid peroxidation by donating a phenolic hydrogen to the free radical. Vitamin C (ascorbic acid) then regenerates Vitamin E by reducing the alpha-tocopherol radical, producing an ascorbic acid radical. Neither of these radicals is particularly reactive due to the energetic stability of their unpaired electrons. Ascorbic acid is in turn

regenerated by glutathione (Fang *et al*, 2002). Lipid hydroperoxides are converted to alcohols by glutathione peroxidase.

These examples are but a few of the many ROS and RNS scavengers present physiologically. A more extensive, but not complete, list includes uric acid (a metabolite of purines), salicylate, mannitol, carotenoid, ubiquinone, bilirubin (a product of haemoglobin catabolism), -lipoate, arginine, citrulline, glycine, taurine, histidine, creatine (a metabolite of arginine, glycine and methionine), carnosine (-alanyl-Lhistidine, which is abundant in skeletal muscle), tetrahydrobiopterin (a metabolite of guanosine triphosphate and thus glutamine), phytate, and tea polyphenols (Fridovich, 1999; Lawler, 2002; Machlin & Bendich, 1987; Wu & Meininger, 2000; Lass *et al*, 2002; Akashi *et al*, 2001; Redmond *et al*, 1996). The key determinant of the degree of oxidative stress is thus the balance between the biological production and subsequent scavenging of ROS.

1.8 Neurodegenerative Diseases

These may be defined as neurological damage accompanied by a loss of neurons. Waldmeier & Tatton (2004) summarise the causes that may trigger neurodegenerative disease (ND) as follows:

Impaired axonal transport	Glutamate receptor over-activation	Excessive levels of ROS
Inadequate provision of trophic molecules by neighbouring neurons or glia	Reduced mitochondrial energy production	Increased formation/inadequate degradation of inappropriately folded proteins
Compromised metabolic pathways	Virus/prion infection	Inflammatory processes

Vogels *et al* (1990) suggest that decreased neuronal cell body size, atrophy of neuronal dendrites and reductions in axonal terminal fields as well as neuronal loss are all factors to consider when explaining ND.

1.8.1 Aging

The Green Paper on Demographic Change, prepared by the European Commission extrapolates that by 2030 the population of Europe over 80 will have nearly doubled from 18.8 million in March 2005 to 34.7 million twenty five years from now – the so-called “baby boomer” generation. And by 2050, the

number of people over 80 years will have increased by 180%. And Europe is not alone – a similar increase in the aging population is found in Australia (www.abs.gov.au/ausstats) and the United States (www.census.gov/population).

This trend does not appear to be reflected in sub-Saharan Africa, where the life-expectancy at birth is 47.1yrs, with the prevalence of AIDS set at 10% (Lahola, 2005). Much debate exists over this figure since AIDS is not a notifiable disease and may not be listed as the cause of death. However research into neuroprotectants is hugely important, as those suffering from AIDS experience related neurological problems such as AIDS dementia which may benefit from research of this kind.

Since the development of neurodegenerative disease is strongly associated with aging, new challenges exist for neuroscientists to develop ways of ameliorating the effects of these debilitating age-related conditions. Free radicals have been shown to play a role in the aging process (Campbell, 1999). A significant correlation exists between DHEA(S) levels and total antioxidant capacity (Petruzzi *et* al, 2002) and it is postulated that the decrease in TAC in elderly subjects is related to changes in antioxidant systems such as DHEA(S). Kimonides *et al* (1998) suggest that since DHEA(S) protects against excitatory amino acid-induced neurotoxicity, lowered DHEA levels may increase the vulnerability of the aging or stressed human brain to neurotoxic glutamate release after cerebral ischaemia, neural insults and neurodegenerative diseases. DHEA has also been identified as a possible avenue for hormone replacement therapy in post-menopausal women since it influences estradiol and estrone serum concentrations (Genazzani *et al*, 2002). Thus, the importance of research into neurodegenerative diseases cannot be underestimated.

The DHEA/cortisol ratio increases with age and this alteration in serum levels may be partly responsible for the catabolic state that occurs with aging. This is in contrast to the situation in the first and second decades when DHEA are relatively high (Baulieu, 1996).

1.8.2 Alzheimer's Disease

In 1907, a German psychiatrist and neuroanatomist published a case study on Auguste D, a patient in her late fifties who exhibited a loss of memory and language ability. He referred to it as a clinical-pathological entity. The disorder is named for Alois Alzheimer and it is the most common of the neurodegenerative disorders of aging (Katzman & Thal, 1989). Today this disorder afflicts millions of people the world over, the largest proportion of whom are over the age of 60.

Alzheimer's disease (AD) is a progressive neurodegenerative condition impairing mental function, memory, reason, language and self-sufficiency (Horsburg & Saitoh, 1994). AD is characterised by the presence of an excessive number of senile or neuritic plaques with -amyloid cores and neurofibrillary tangles comprised of Tau proteins, as well as a selective distribution of cell loss. These do not occur throughout the brain, but in several areas including the hippocampus where the large pyramidal cells in the CA1 and subiculum are most affected (Katzman & Thal, 1989).
Katzman and Thal (1989) describe the clinical presentation of AD as a progressive intellectual deterioration that involves memory, orientation, language functions, as well as higher brain function including personality, judgement, calculation, visual-spatial and constructional abilities.
AD is accompanied by a loss of neurons in the amygdala (Vereecken *et al*, 1994), cerebral cortex and hippocampus (Beers & Berkow, 1999) and cell shrinkage as well as neuron loss in the nucleus raphes dorsalis (Aletrino *et al*, 1992) and the nucleus basalis of Meynert complex (Vogels *et al*, 1990). Van Rensburg *et al* (1994) have also demonstrated that lipid peroxidation is key in the aetiology of age-related diseases such as AD.
In areas in which -amyloid plaques and neurofibrillary tangles are found, the enzyme responsible for the production of acetylcholine is reduced and thus the transmission of ACh is affected. This is accompanied by degeneration of cholinergic neurons (Bannister, 1992). The soluble oligimer of the -amyloid peptide has been demonstrated to activate microglia – which are important in the neuroinflammation associated with AD (Streit *et al*, 2004) – as well as inducing QA production in cells (Guillemin *et al*, 2005).
It is widely recognised that AD accounts for almost two thirds of senile dementia cases, giving it the title of most common cause of dementia late in life (Katzman & Thal, 1989). The incidence of AD increases with age (Beers & Berkow, 1999).

Cognitive decline is inevitable in AD, since AD cannot be cured with current medical knowledge. All treatments are symptomatic and although drugs which enhance cholinergic transmission modify the loss of memory experienced in early stages, they cannot prevent the deterioration of the fundamental pathology. AD has been described as a multineurotransmitter deficiency disease, and although the reduction of acetylcholinesterase in the most well documented, serotonin, noradrenalin, somatostatin, and corticotropin-releasing factor are also reduced (Markesbery, 1997).

The acetylcholinesterases enhance central cholinergic function by reversibly inhibiting the enzyme acetylcholinesterase (AChE). This enzyme is responsible for the degradation of acetylcholine (ACh) in the synaptic cleft, and its inhibition results in an increased half-life of Ach and an enhanced post-synaptic signal (Gibbon, 2003).

A correlation exists between decreased levels of steroids and increased levels of important proteins implicated in the development of neuritic plaques and neurofibrillary tangles, which supports the theory that neurosteroids may be protective in AD (Weill-Engerer *et al*, 2002) and iron accumulation in AD is a source of redox-generated free radicals (Smith *et al*, 1997), suggesting that antioxidant properties may be of value. Free radicals are involved in the pathogenesis of neuron death, but since tissue injury also results in free radical generation, it is still unclear whether these increased free radicals are a cause or effect of the disease (Markesbery, 1997).

Cerebrospinal fluid levels of QA correlate well with severity of neurological deficits, suggesting QA plays a direct role in the progression of neurodegenerative disorders (Takikawa, 2005). The tryptophan-kynurenine pathway has been demonstrated to be up-regulated in AD, thereby leading to increased QA synthesis (Guillemin *et al*, 2005).

It is well documented that AD patients exhibit raised levels of iron (Goodman, 1953, Ehmann *et al*, 1986), which participates in the Fenton reaction to liberate free radicals as shown above, which can act as lipid peroxidants.

1.9 DHEA

DHEA, an intermediate hormone in the synthetic pathway from cholesterol to the sex steroids testosterone and oestrogen, has risen from obscurity. Now widely referred to as a "fountain of youth" or even an "unknown star" (Schlienger *et al*, 2002), this hormone has gained new respect in scientific circles. Adolf Butenandt was awarded the Nobel Prize for his work on the sex steroids, part of which included isolating DHEA from urine by acid hydrolysis in the early 1930's (Butenandt & Dannenbaum, 1934). Its sulphated form DHEAS was first identified ten years later by Munson and colleagues (1944). It once again came to the fore almost two decades later when Baulieu (1960) discovered that it was synthesised by the adrenal cortex.

DHEA is a multifunctional, pleiotropic hormone whose effects are mediated through nongenomic actions on various neurotransmitter systems. The hormone increases neuronal excitability, modulates neuronal plasticity and exhibits neuroprotective properties (Wolf & Kirschbaum, 1999).

A ScienceDirect (www.sciencedirect.com) search for the keyword "dehydroepiandrosterone" yields just over 7 330 hits in the database over all years. Of these, over thirty percent were published with the last decade, and almost fifteen per cent within the last two years, showing how interest in this hormone has grown substantially.

DHEAS functions as a circulating hydrophilic storage form or reservoir for DHEA. Since the latter is more lipophilic, it may be converted intracellularly to androgens and oestrogens as shown in Fig 1.12 below. The ratios of circulating DHEAS to DHEA are 500:1 and 250:1 in men and women, respectively (Labrie *et al*, 1997). This ratio can be explained by considering the metabolic clearance rates (MCR) and half-lives of these two compounds. The MCRs are 2000L/day and 13L/day for DHEA and DHEAS, respectively and half-lives of DHEA and DHEAS range from 30 minutes to 7 hours and 10 hours to 24 hours (Frye *et al*, 2000; Rosenfeld *et al,* 1975). Another important factor is the weak protein-binding displayed by DHEA, while its sulphate derivative is more strongly bound (Kroboth *et al*, 1999).

Research using exogenously administered DHEA has demonstrated that DHEA and DHEAS are constantly undergoing interconversion (Arlt *et al*, 1998; Arlt *et al*, 1999) and Bird and co-workers (1984) established that the conversion ratios for the conversion of DHEAS to DHEA were 0.006 in males and 0.004 in females, confirming that a substantial amount of DHEA derives from DHEAS.

Sulphation and glucuronidation are the main conjugation pathways of Phase II metabolism. Sulphation of lipophilic moieties increases their water solubility - enhancing the ability of the body to transport and excrete these molecules. It is well established that sulphation is a major reaction in steroid synthesis, transport and metabolism.

Concentrations in the brain are somewhat different with Liere *et al* (2000) demonstrating that the concentration as assayed by gas chromatography-mass spectrometry (GC-MS) in the rat brain of the unconjugated neurosteroid is 0.45 ± 0.02ng/g , while that of the sulphate ester is 2.47 ± 0.27ng/g. Corpéchot *et al* (1981) show that in the rat brain, the concentration of DHEAS is up to 20 times that of the plasma, and exceeds concentrations in organs such as the liver, kidneys and adrenals.

The study indicated that the biosynthesis of C-19 steroids does occur at a low rate in rat adrenals and the authors suggested in 1981 that DHEAS was produced or accumulated in the rat brain.

1.9.1 The Structure of DHEA

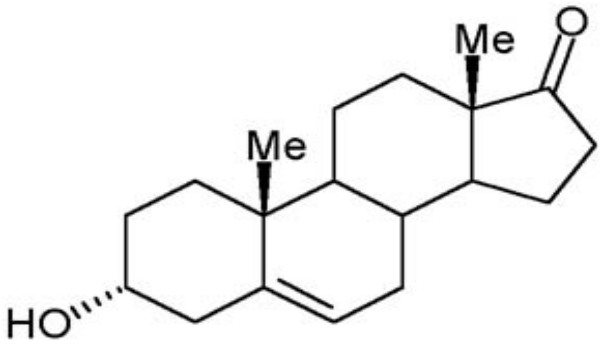


Fig 1.11 The structure of 5-androsten-3-β-ol-17-one, otherwise known as dehydroepiandrosterone.

Dehydroepiandrosterone (DHEA) is the steroid with the highest circulating serum concentration, ensuring that sufficient substrate exists for biotransformation into androgens and estrogens in peripheral tissues (Labrie *et al*, 1998). Both DHEA and pregnenolene, the immediate precursor hormone to DHEA, are classed as 3β-hydroxy-Δ5 compounds. DHEA itself can be referred to as a precursor hormone, due to its metabolism to the important sex steroids 17-β-estrodiol and testosterone (see Fig 1.12). This fact has caused some scientists to refer to DHEA as a "mother hormone", although Baulieu (1996) argues that this is inappropriate. DHEA has a three- to ten-fold predominance of androgenic over oestrogenic activity, although it gives rise to a substantial quantity of both androgens and oestrogens (Kroboth *et al*, 1999).

The role of DHEA in the periphery has not been elucidated beyond its role as a precursor hormone in sex steroid biosynthesis.

1.9.2 Physical Properties of DHEA

It is a fine white or almost white crystalline powder which is freely soluble in alcohol, yet practically insoluble in water. It melts between 146°C and 151°C. It should be stored in a cool dry place and protected from light. Its molecular formula is $C_{19}H_{28}O_2$ and its relative molecular mass is 288.4g/mol (European Pharmacopoeia).

1.9.3 Biosynthesis

The human adrenal gland was identified by Bartholomeus Eustachius in 1563 (Bourne, 1949). In mammals it consists of a centrally placed medulla and peripheral cortex. The main body of the cortex consists of columns of cells making up the zona fasciculate (ZF), as well as a more specialised area adjacent to the medulla which forms the zona reticularis (ZR). These morphologically distinct zones, along with the outer zona glomerulosa (ZG), comprise the adrenal gland (Hornsby, 1999).

Dehydroepiandrosterone, with its sulphated ester, DHEAS are the major adrenal steroid hormones. The ZR, which develops at adrenarche, is the site of DHEA(S) biosynthesis in the adult human adrenal cortex (Endoh *et al*, 1996). Fig 1.12 below illustrates the adrenal biosynthesis of DHEA. DHEA secretion is stimulated by release of adrenal corticotrophin releasing hormone (ACTH); the adrenal synthesis of DHEAS is also stimulated by the hormone but normally no daily fluctuations are visible due to the much lower MCR (Baulieu, 1996). In healthy females DHEA(S) is synthesised exclusively by the adrenal cortex, while in males as much as 25% may be secreted by the testes (Kroboth *et al*, 1999).

It has already been established that DHEA concentrations in peripheral blood exhibit a circadian rhythm (Rosenfeld *et al*, 1975), and that this rhythm is attenuated with age, while that of cortisol (also produced in response to stimulus by ACTH) is largely unaffected by the aging process (Parker *et al*, 2000). Additionally, Ceserini *et al* (2000) determined that no significant DHEA circadian rhythm existed in older subjects, but a single 50mg DHEA dose restored rhythmicity and increased serum concentrations, thereby favourably affecting the DHEA/cortisol ratio.

There is a wide inter-individual variability in DHEA(S), even when variables such as age and apparent health are taken into account (Berr *et al*, 1996), but the concentration serves as a highly specific and reliable marker in individuals (Orentreich *et al*, 1992), supporting the idea that a genetic component to the variation exists (Rotter *et al*, 1985).

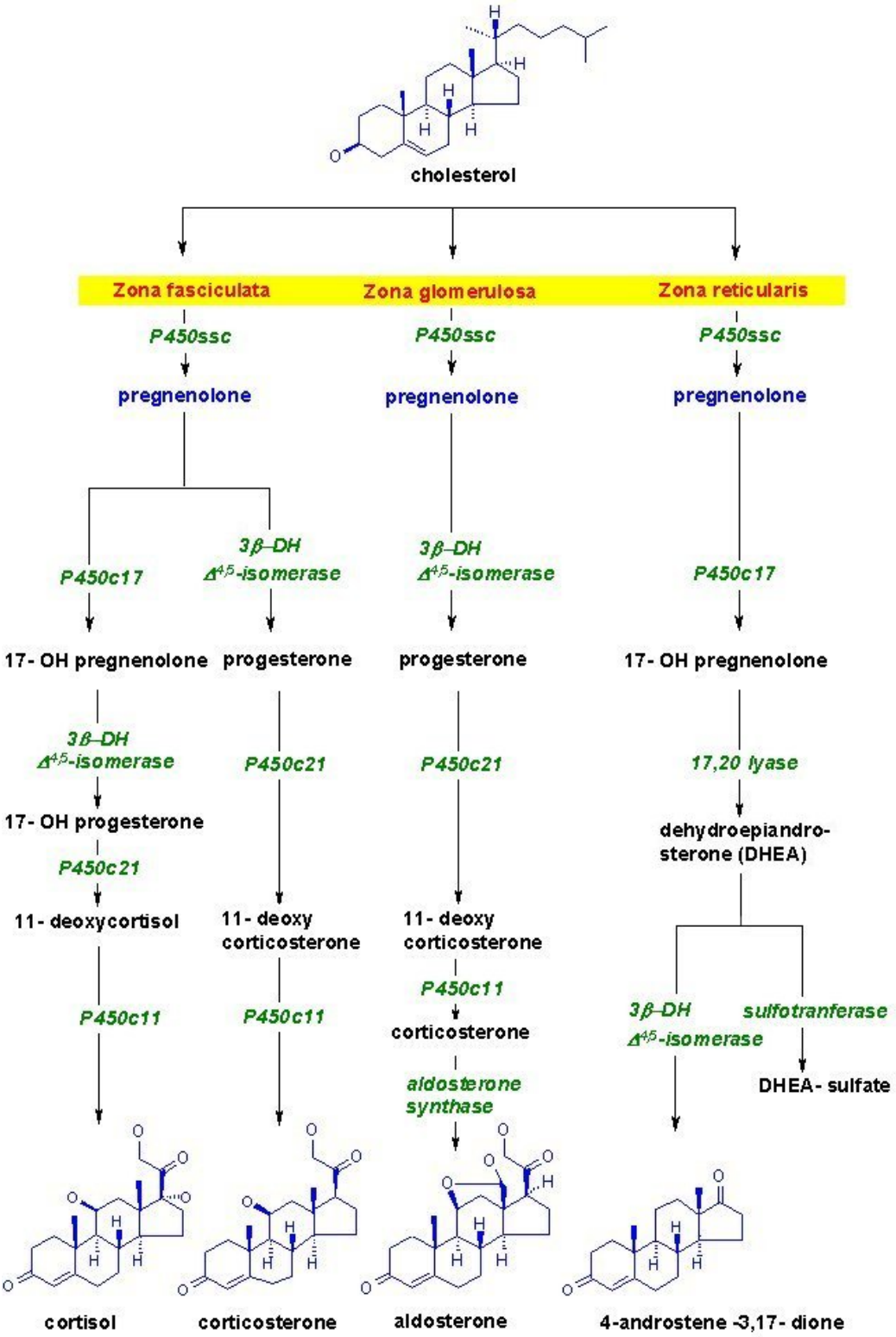


Fig 1.12 This diagram depicts the site of synthesis in the adrenal glands of both DHEA and its sulphated derivative, DHEAS. The relevant enzymes in the conversion from cholesterol are also indicated (www.med.unibs.it/~marchesi/sterhorm.html).

1.9.4 Distribution of DHEA

While circulating levels of DHEAS are 100 times that of DHEA, in the cerebrospinal fluid the ratio falls to about 3 (Guazzo *et al,* 1996). It is the most prevalent steroid of the brain in rats and humans (Majewska, 1995). Robel and Baulieu (1995) reported brain:plasma ratios of 4 and 8.5 for DHEA and DHEAS, respectively. DHEA is weakly bound to sex hormone-binding globulin (SHBG) and to albumin (Dennehy & Tsourounis, 1999). Its sulphate ester is more strongly bound, resulting in higher clearance values and a longer half-life for DHEAS as mentioned in Section 1.9.

1.9.5 The Metabolism of DHEA

As previously discussed, DHEA is a precursor steroid. Its synthesis from cholesterol *via* pregnenolene is catalysed by cytochromes P450 (Doostzadeh & Morfin, 1996) and is shown in Fig 1.13 below. DHEA and its sulphate ester exist in equilibrium. Unconjugated DHEA may be converted to both oestrogens and androgens. The human steroidogenic enzyme P450c17 converts little 17α-hydroxyprogesterone to androstenedione in the periphery. Androstenedione synthesised from DHEA via 3β-hydroxysteroid dehydrogenase is in turn converted to testosterone and oestradiol by isozymes of 17β-hydroxysteroid dehydrogenase and by P450 aromatase, respectively. Thus, the biosynthesis of all sex steroids proceeds through DHEA (Allolio & Arlt, 2003).

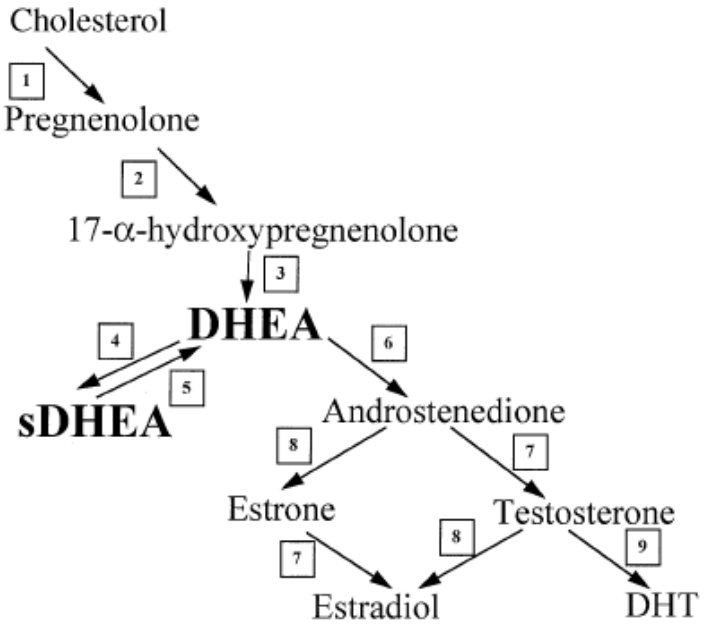


Fig 1.13 The diagram illustrates the biosynthesis and metabolic conversion of DHEA. The enzymes catalysing each step are numbered as follows: 1: P450scc (20,22

desmolases); 2: P450c17 (17α-hydroxylase); 3: P450c17 (17-20 desmolase); 4: sulfotransferase; 5: sulfatase; 6: 3-β-hydroxysteroid dehydrogenase, 7: 17-β-hydroxysteroid dehydrogenase, 8: aromatase, 9: 5-α-reductase. DHT: dihydrotestosterone (Cormier *et al*, 2001).

As is visible in Fig 1.13, the enzymes responsible for the equilibrium between DHEA and its sulphate ester are steroid sulphatase, which catalyses unconjugated steroid formation, as well as neurosteroid sulfuryl transferase which in turn catalyses the formation of the sulphate ester. In primates, studies characterising the specific activity of the steroid sulphatase have produced conflicting results with regard to the influence of gender (Lakshmi *et al*, 1981; Kříž *et al*, 2005). Kříž and coworkers showed that the specific activity of steroid sulphatase declined in the female brain in the order cerebellum, cortex, subcortex while no major changes were observed between the regions of the male brain. The sulphotransferase was established in nine regions of the rat brain, with the highest concentrations observed in the hippocampus, frontal cortex, thalamus and basal ganglia – all four of which are regions involved in the control of coordination and memory (Aldred and Waring, 1999). The authors note that the enzyme activity in the adult rat brain is a quarter of that found in the liver and a third of that in the kidney.

It is suggested that the modulation of neurotransmission may be through direct action on the neuronal membrane (Aldred & Waring, 1999) and this is supported by the fact that neurosteroids can cause structural changes in neurons and astrocytes (Schumacher *et al*, 1996). Aldred & Waring (1999) also observed that the brain regions with the highest sulphotransferase activity correspond with the regions of highest DHEA biosynthesis in previously published results by Robel *et al* (1991). Orally administered DHEA undergoes minimal bioconversion due to first pass metabolism (Callies *et al*, 2000).

1.9.6 DHEA Undergoes an Age-related Decline

DHEA has been referred to as the "fountain of youth" or an "unknown star". This can be attributed, amongst other factors, to its well documented age-related decline. DHEA levels have been shown to peak in the third decade in both males and females. Levels post-natally are initially very high, due to extensive production by the foetal adrenals. Levels rapidly drop as the foetal zone regresses and

minimal changes in serum concentration occur until the adrenarche. ZR cells, which up until this stage have not been present in significant quantities, begin to synthesise DHEA(S).

The low expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) demonstrated by Gell *et al* (1996), Parker (1997) and Endoh *et al* (1996) plays a role since the inhibition of 3β-HSD results in increased production of DHEA in human and bovine models (Hornsby, 1980). At adrenarche 3β-HSD expression in the ZR is suppressed, accounting for the substantial increase in DHEA(S) levels and as children age (Gell *et al*, 1996). This can be explained since 3β-HSD competes with P450c17 for pregnenolene.

Serum levels increase to peak values in the third decade, after which an asymptotic decline is then apparent. Levels in the 70-79 year old population are reported to be approximately 20% of peak values (Orentreich *et al*, 1984). These cross sectional data were confirmed by a longitudinal study in normal healthy males (Orentreich *et al*, 1992).

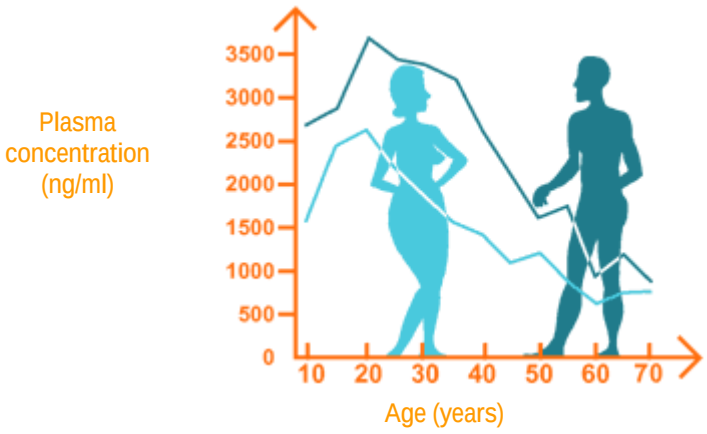


Fig 1.14 The graph, modified from Orentreich *et al* (1984), illustrates the distinct age-related decline of DHEA in both male and female human subjects.

Endoh *et al* suggest that the most straightforward hypothesis for this age-related decline in synthesis by the adrenal glands is a progressive decline in zona reticularis cells in the adrenal cortex, possibly due to a greater susceptibility to apoptosis, which in turn is due to differences in gene expression between ZR and ZF cells (Khoury *et al*, 1987, Khoury & Berline, 1988). Hornsby (1999) concurs with

the hypothesis that a loss of ZR cells is responsible for the decrease in adrenal androgen biosynthesis.

A large inter-individual variation in DHEA(S) levels exists and when age is factored out of the equation, the residual variation is shown to have a significant genetic component (Rotter *et al*, 1985). Circulating hormones have an impact on 3HSD levels in the cells of the reticularis, thus regulating DHEA(S) biosynthesis in this region (Endoh *et al*, 1996).

Morley *et al* (1997) demonstrated that the amount of bioavailable testosterone undergoes a similar age related decline due to the reduced levels of the precursor DHEA(S).

In elderly human subjects, a once daily dose of DHEA (50mg) restored endogenous DHEA to peak youth levels, and subsequently increased the levels of circulating androgens and estrogens (Arlt *et al*, 1998; Arlt *et al*, 1999).

Cytochrome P450 17c catalyses the hydroxylation of pregnenolene and its sulphate ester, as well as the subsequent removal of the side chains of these hydroxylated products (see Fig 1.12). The lyase activity which results in cleavage of the side chain declines with age, resulting in less DHEA and subsequently less testosterone (Morley *et al,* 1997).

Morley and colleagues (1997) suggest that DHEAS binds to albumin and then forms a tertiary complex with testosterone, enabling the targeting of specific receptors for a faster action at the cellular level.

In addition to aging, the levels of DHEA(S) are affected by physiological condition. The concentrations of both DHEA and its sulphated form are reduced during the progression of the HIV infection (Christeff *et al*, 1992; Grinspoon & Bilezikian, 1992). In a murine AIDS model, Zhang *et al* (1999) showed that DHEA affected immune dysfunction and prevented hepatic lipid peroxidation. This murine retrovirus has been demonstrated to be remarkably similar to HIV (Liang *et al*, 1996). Robinzon & Cutolo (1999) argue that the replacement of DHEA may be of value with glucocorticoid therapy as it ameliorates the deleterious effects of the latter.

1.9.7 Receptor for DHEA

To date, no specific DHEA receptor has been identified in the plasma membrane, nucleus or cytoplasm (Kaasik *et al,* 2003), although the hormone has been shown to interact nonspecifically with receptors in murine and human t-lymphocytes which also effectively bind dihydrotestosterone (Allolio & Arlt, 2003). DHEA has been shown to be a negative modulator of $GABA_A$ (Majewska *et al*, 1990),

possibly through the picrotoxin site (Sousa & Ticku, 1997), a positive modulator of NMDA receptors (Wu *et al*., 1991) and a sigma1 receptor agonist (Romieu *et al*, 2003).

1.9.8 The Myriad Effects of DHEA

1.9.8.1 DHEA and General Well-being

Supplementation of DHEA in the elderly has resulted in observations of improved well-being, determined by factors such as improved quality of sleep, increased energy, a greater sense of relaxation, and an improved ability to handle stress (Morales *et al*, 1994), irrespective of gender. This is supported by Glei *et al* (2004) who furthermore observed that higher DHEA levels may be associated with better self-rated health. An increase in libido has also been reported in the DHEAge Study by Baulieu *et al* (2000), as well improved skin status in terms of hydration, epidermal thickness, sebum production and pigmentation.

1.9.8.2 DHEA and Depression

Wolkowitz *et al* (1997) identified older patients with DSM-III-R Major Depression and Hamilton Depression Rating Scale ratings of 17 out of 21 or higher. These patients, after being medication free for six weeks, received DHEA open label for four weeks and improved significantly in all psychiatric rating scales, specifically in the areas of cognitive disturbance and verbal memory. The authors acknowledge the limitations of their study (small sample size) but suggest that DHEA has antidepressant and cognition-enhancing effects in middle-aged and elderly patients with major depression. Ritsner *et al* (2003) demonstrated the cortisol/DHEA ratio is raised in patients with schizophrenia, and that when variables such as age, illness and age of onset were controlled this elevated ratio correlated with depression, trait anger and hostility.

1.9.8.3 DHEA and Diabetes

It has been demonstrated that when DHEA is administered to streptozotocin-treated rats, the pattern of both nonenzymatic (e.g. reduced glutathione) and enzymatic (superoxide dismutase, glutathione peroxidase and catalase) antioxidants returns to near control values (Aragno *et al*, 1999), implying that DHEA administration may counteract the perioxidative processes involved in diabetic complications.

1.9.8.4 DHEA and Obesity

DHEA has been shown to have effects against obesity (Bellino *et al*, 1995; Kalimi & Regelson, 1990). In addition to this, DHEA has been demonstrated to exert a synergistic anorectic effect when administered with *d*-fenfluramine, a pharmacologic agent which affects serotonin synthesis and re-uptake in the hypothalamus (Gillen *et al*, 1999). Rats receiving DHEA supplementation consume fewer total calories, hypothesised by Pham *et al* (2000) to be due to the acute release of norepinephrine, epinephrine and dopamine in select regions of the hypothalamus.

1.9.8.5 DHEA and the Cardiovascular System

Bonnet *et al* (2003) have demonstrated that 30mg/kg every two days results in the prevention of an increase in pulmonary artery hypertension, cardiac right ventricle hypertrophy and pulmonary artery remodeling in a chronic hypoxic-pulmonary hypertension model in rats. Additionally, hypertensive rats experienced a rapid decrease in pulmonary artery hypertension after oral or intravascular injection. Furthermore, DHEA does not affect systemic pressure, cardiac outflow, left ventricle contraction, and heart rate, suggesting a specific activity of DHEA on pulmonary circulation.

1.9.8.6 DHEA and Bone Mineral Density

Several independent studies on the effects of DHEA on bone mineral density (BMD) and bone turnover markers have shown a positive correlation with DHEA supplementation in elderly subjects (Labrie *et al*, 1997, Baulieu *et al*, 2000, Villareal *et al*,). DHEA supplementation results in a fairly rapid increase in BMD as well as serum osteocalcin concentration, an established marker of bone formation. This is accompanied by a decrease in urinary hydroxyproline excretion, indicating a decrease in bone resorption (Labrie *et* al, 1997). Cormier *et al* (2001) suggest that the ability of DHEA to improve bone mineral density appears to be related to both these factors, but suggest that this may, however, be due to conversion to the sex steroids, rather than attributable to DHEA itself. A number of elderly patients in a DHEA replacement trial independently reported an improvement in pre-existing joint pains and mobility (Morales *et al*, 1994).

1.9.8.7 DHEA and the Immune System

DHEA has been identified as a therapeutic agent for the treatment of the chronic autoimmune rheumatic disease of unknown aetiology Systemic Lupus Erythematosus (SLE). Norman (2001)

outlines the clinical trials indicating that oral administration of DHEA in the form of GL-701, a pharmaceutical preparation, resulted in a reduction in the rate of SLE exacerbations, as well as allowing a reduction in the concurrent steroid dosage in patients.

DHEA significantly elevated hepatic phospholipids in retrovirus infected mice and was shown to significantly increase vitamin E levels when administered in combination with melatonin. Both these levels were shown to significantly decline with retrovirus infection (Zhang *et al*, 1999). The authors concluded that DHEA and melatonin treatment significantly prevented immune dysfunction, excessive lipid peroxidation and loss of tissue vitamin E induced by retrovirus infection (Zhang *et al*, 1999).

Low levels of DHEA have been established in patients with breast (Zumoff *et* a, 1981) and prostrate (Stahl *et al*, 1992) cancer. Additionally, the anti-oncogenic properties of DHEA have been demonstrated in animal models (Schwartz *et al*, 1986).

1.9.8.8 DHEA and Melatonin

DHEA(S) and melatonin have several effects in common, namely immune system enhancement, life span prolongation and cancer prevention (Milewich *et al*, 1995; Reiter, 1998; Agrasal *et al,* 2001). Haus *et al* (1996) showed that murine adrenals incubated with melatonin caused a dose-dependant increase in production and/or secretion of DHEA. The authors suggest that the age-related decline of DHEA and melatonin may be partially due to a decrease in mutual stimulation, thus studying this interaction may be of value in light of the potential neuroprotective characteristics of these hormones. It is suggested that the pineal gland may modulate adrenal cortical function and therefore play a role in the age-related decline of DHEA secretion (Haus *et al*, 1996).

1.9.8.9 DHEA and Aging

It has already been established that DHEA serum levels undergo an age-related decline (see Fig 1.14, Orentreich *et al*, 1984). Thus the restoration of these serum levels to levels found in younger subjects, as well as the resultant effect on neuroendocrine metabolic functions is an area of interest. Morales *et al* (1994) found that 50mg DHEA daily, administered orally was sufficient to achieve the desired result, and that levels were raised from placebo values within two weeks. The topic of DHEA supplementation in the elderly was explored in the DHEAge Study, which was conducted to establish how and when DHEA should be administered. Baulieu *et al* (2000) suggests that in addition to examining supplementation in the aging population due to a physiological decline in concentration,

studies could be conducted with a "pathological end-point" in mind. The relative risk of death for men was reported to be highest with lowest levels of DHEAS and low levels of DHEAS proved to be a reliable predictor of death in male smokers (Mazat *et al*, 2001).

The administration of steroids may be a pharmacological route to the amelioration of age-related changes in the CNS for the following reasons:

1 Steroids are important in the functioning of both the central and peripheral nervous systems.
2 Several steroids have been suggested as neuroprotectants.
3 Several neuroactive steroids undergo an age-related decline.
4 Due to their lipophilic nature, unconjugated steroids readily cross the blood-brain barrier and are accumulated within the brain (Schumacher *et al*, 2003).

1.9.8.10 DHEA and Alzeimer's Disease

Research regarding the link between DHEA(S) levels and AD is unclear. Low DHEAS levels are risk factors for AD (Hillen *et al*, 2000) when human subjects were matched for age, gender, morbidity and immobility, while Berr and colleagues did not observe lowered DHEAS levels in subjects with prevalent dementia or AD.

However, Brown *et al* (2003) have demonstrated that DHEA levels in the brain and cerebrospinal fluid are raised in patients with AD relative to age-matched controls, contrasting with situation of decreased serum levels, suggesting the CSF and serum levels of DHEA can be used diagnostically in the examination of AD neuropathology. The addition of ferrous sulphate to sera of controls caused an increase is DHEA, which was not observed in sera from AD patients (Brown *et al*, 2003).

Brown *et al* (2003) showed that although DHEA levels were increased in AD patients, immunoreactivity to P450c17 was not evident, indicating that an alternative pathway for the synthesis of DHEA exists in the brain, and that this pathway is activated by increased oxidative stress. The authors also suggest an alternative precursor which may be use to identify AD closer to the onset of the disease, or establish the progression of AD. The question remains whether DHEA has a role in the pathology of the disease, or whether it is merely an epiphenomenon.

1.9.9 Neurosteroids and Neuroactive Steroids

The term "neurosteroid" was coined by Etienne-Emile Baulieu in 1981 for a group of hormones with certain properties. Robel and Baulieu (1998) explain that neurosteroid refers to steroids which accumulate in the brain independently of endocrine glands and undergo *de novo* synthesis within the nervous system. These steroidal compounds must also exert physiological/pharmacological actions on neuronal cells in central and peripheral nervous system (Gasior *et al*, 1999). Although the synthesis of DHEA in the zona reticularis has already been mentioned, the hormone is entitled to be termed a neurosteroid due to its ability to undergo *de novo* synthesis in the brain, independently of the peripheral endocrine pathway detailed above (Aldred & Waring, 1999).

Additionally, DHEA is a neuroactive steroid, as DHEA synthesised in the adrenal gland has been demonstrated to have an influence in the CNS. This neurosteroid exerts neurotrophic effects with extensions in the processes of neurofilament-positive neurons and glial fibrillary acid protein-positive astrocytes (Roberts *et al*, 1987). Compagnone and Mellon (1998) provided evidence that DHEA selectively increases the length of neurites with the axonal marker Tau-1, while DHEAS increased the length of those with the dendritic marker MAP-2.

Oligodendrocytes and astrocytes are able to produce DHEA *via* an alternative pathway that is independent of P450c17 and that the oxidative environment of the brain may have an effect on glial, but not neuronal, formation of DHEA (Brown *et al,* 2000). The authors suggest this may be influenced by elevated levels of endogenous ROS.

Steroidogenic enzymes including cytochrome P450 cholesterol side-chain cleavage (P450scc), aromatase, 5α-reductase, 3α-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase have been identified in the brain through molecular and biochemical investigations (Stoffel-Wagner, 2001).

DHEA was first recognised as an adrenal steroid hormone (Baulieu, 1960) but researchers have realised in the half-century since the afore-mentioned discovery that both adrenal and gonadal hormones are not merely responsible for reproduction, but these hormones may be neurally important (Schumacher and Robert, 2002; Schumacher *et al*, 2000) since free steroids are sufficiently lipophilic to cross the blood-brain barrier.

The panicogenic agent pentagastrin causes the release of neuroactive steroids such as DHEA and allopregnenolene (Tait *et al*, 2002), although the reason for this is undetermined.

1.9.10 DHEA and Neuroprotection

DHEA decreases the neurodegeneration caused by 1-methyl-4-phenylpyridium (MPP+) induced neurotoxicity in the rat nigrostriatal dopaminergic system (Tomas-Camardiel *et al*, 2002). Estradiol and testosterone were also tested, though only the former was found to be protective. The authors suggested that DHEA exerts its neuroprotective effect by partially preventing the MPP+ induced inhibition of NADH oxidase activity, while estradiol was thought to reduce the uptake of the toxin into the dopaminergic neurons. DHEA is effective in reducing the catecholeamine depletion associated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration (D'Astous *et al,* 2002) and the investigators conclude that DHEA is neuroprotective of dopaminergic (DA) neurons.
DHEA is able to completely reverse the age-related significant decrease in corticotrophin-releasing hormone in the hypothalamic paraventricular nucleus, which may be correlated with general impairment of cerebral activity. This result may be due to the estrogenic influence of DHEA on CRH gene expression but it is unclear whether DHEA or its metabolites are responsible or whether the regulation may be influenced by a more indirect action (Givalois *et al*, 1997). However, Suzuki *et al* (2004) demonstrated that while DHEA stimulated human neural stem cell growth and affected the number of cells labelled with BrdUrd, neither pregnenolene, nor six other steroids found in the metabolic pathway of DHEA had any effect.

Neurons in culture experience improved survival and differentiation when exposed to DHEA(S) in the nanomolar range (Bologa *et al*, 1987). Human neural stem cells exposed to DHEA undergo more neurogenesis after differentiation (Suzuki *et al*, 2004) and the hormone positively regulates the number of neurons produced in culture from human neural stem cells. This is the first steroid tested to have such a marked effect.

DHEA's action is dose-dependent as Mastrocola and colleagues (2003) demonstrated that while DHEA was acting in an antioxidant capacity at lower doses, at high tissue concentrations, it acted in a pro-oxidant manner. At doses 50mg and above daily for seven days, the neurosteroid doubled the level of hydrogen peroxide. Doses below 50mg daily had no effect. The antioxidant effect of DHEA at low doses was confirmed (Mastrocola *et* al, 2003).

DHEA increased neuronal survival after anoxia and Marx and colleagues (2000) established that this effect was not due to metabolite formation. DHEA inhibited the toxic effects of glutamate on hippocampal neurons in a steroid specific and dose-dependent manner (Cardounel *et al*, 1999) and has been demonstrated to ameliorate the effects of EAA-induced neurotoxicity in both *in vitro* and *in vivo* models using NMDA (Kimonides *et al*, 1998). DHEA has memory enhancing effects in mice (Flood *et al*, 1992, Flood & Roberts, 1988).

DHEAS exerted a substantial neuroprotective effect in reversible spinal cord ischaemia model, but this neuroprotection was time dependent, suggesting that DHEAS may be modifying a rapid process initiated shortly after the start of ischaemia, or modulating a neurotransmitter function (Lapchak *et al*, 2000).

1.9.11 Mechanism of Action of DHEA

A specific mechanism of action has not been identified for DHEA but the following provide a brief summary of the proposed mechanisms and hypotheses.

Garthewaite & Garthewaite (1986) hypothesise that DHEA(S) exerts it neuroprotective effect by altering Ca^{2+} homeostasis after exposure to NMDA – either by reducing Ca^{2+} entry into cells, or by modifying free Ca^{2+} intracellularly. May *et al* (1990) suggest that its neuroprotection is attributable rather to its nature as a glucocorticoid antagonist. Cardounel *et al* (1999) provided evidence for the down-regulation of glucocorticoid receptors when DHEA was shown to protect hippocampal cells of the HT-22 line against the excitatory amino acid glutamate.

It has been shown that DHEA is protective of cultured neurons even when added an hour after NMDA insult, giving investigators reason to suggest that DHEA interfered with an unidentified process downstream of the initial action of NMDA (Kimonides *et al*, 1998).

Researchers have suggested that the neuroprotective effects of DHEA are attributable to its role as a precursor hormone. Berr *et al* (1996) suggest that the partial transformation to sex steroids, increase of bioavailable insulin like growth factor I and effects on neurotransmitter receptors may be important in the mechanism of action of DHEA (Berr *et al*, 1996; Hiroi *et al*, 2005). The inhibition of the neuroprotective effects of DHEA against kainic acid in rats by administering an aromatase inhibitor caused investigators to suggest that these neuroprotective effects may be mediated, at least in part, by aromatase-facilitated conversion to estradiol (Veiga *et al*, 2003). Jellinck *et al* (2001) observed

that the conversion rate of DHEA to androgens and estrogens was low and postulated that two major metabolites, 7α- and 7β-hydroxydehydroepiandrosterone (7α-OHDHEA, 7β-OHDHEA) may be the focus for further research. These authors maintain that the hydroxylation of DHEA is not a step towards inactivation and elimination since DHEAS is readily produced, but rather the hydroxylated metabolites may have some other role (Jellinck *et al*, 2001) which may be related to the neuroprotective ability (Morfin & Starka, 2001).

An antioxidant mechanism for DHEA has also been proposed (Aragno *et al,* 1999) as outlined in Section 1.9.8.3 above.

Since Lapchak *et al* (2000) have shown that bicuculline, a GABA antagonist, attenuates the neuroprotective effects of DHEAS, it is suggested that its mechanism of neuroprotection is through the enhancement of GABAergic neurotransmission.

Weill-Engerer *et al* (2003) have demonstrated that a trend towards a negative correlation between the density of cortical amyloid deposits and the synthesis of the DHEA metabolites 7α -OH-DHEA (in the frontal cortex) and ADIOL (in the hippocampus) may constitute a neuroprotective role. They believe it is plausible that this metabolism regulates cerebral DHEA availability. DHEA, as well as pregnenolene, have been demonstrated to be protective against amyloid-peptide induced neurotoxicity and amnesia (Cardounel *et al*, 1999; Maurice *et al,* 1998). Since membrane fluidity is a necessary component for the function of signal transduction, DHEA may work to increase membrane fluidity which should facilitate lymphocyte function (Zhang *et al*, 1999).

Lipid Peroxidation Investigation

2.1 Introduction

In the thirties, J. F. Danielli and H. Davson proposed that biological cell membranes were in fact phospholipid bilayers, consisting of two adjacent layers of phospholipids oriented with their hydrophobic ends interacting. In 1972, S. J. Singer and G. L. Nicolson expanded on this theory with their Fluid Mosaic Model, suggesting that proteins are a vital part of these membranes, either as peripheral or integral proteins (Keeton, Gould, 1993). As shown in Fig 2.1, these dynamic phospholipid bilayers consist largely of lipids. When these polyunsaturated lipids experience oxidative stress, this affects the way in which the oxidised molecules interact with other molecules in the phospholipid bilayers, thereby affecting the integrity of the cell. The oxidative destruction of polyunsaturated fatty acids (PUFAs) is referred to as lipid peroxidation. This lipid peroxidation can be especially destructive since chain reactions may be initiated (Reiter *et al*, 1996). Membranes are particularly vulnerable due to their high phospholipid count (Fraser, 1985).

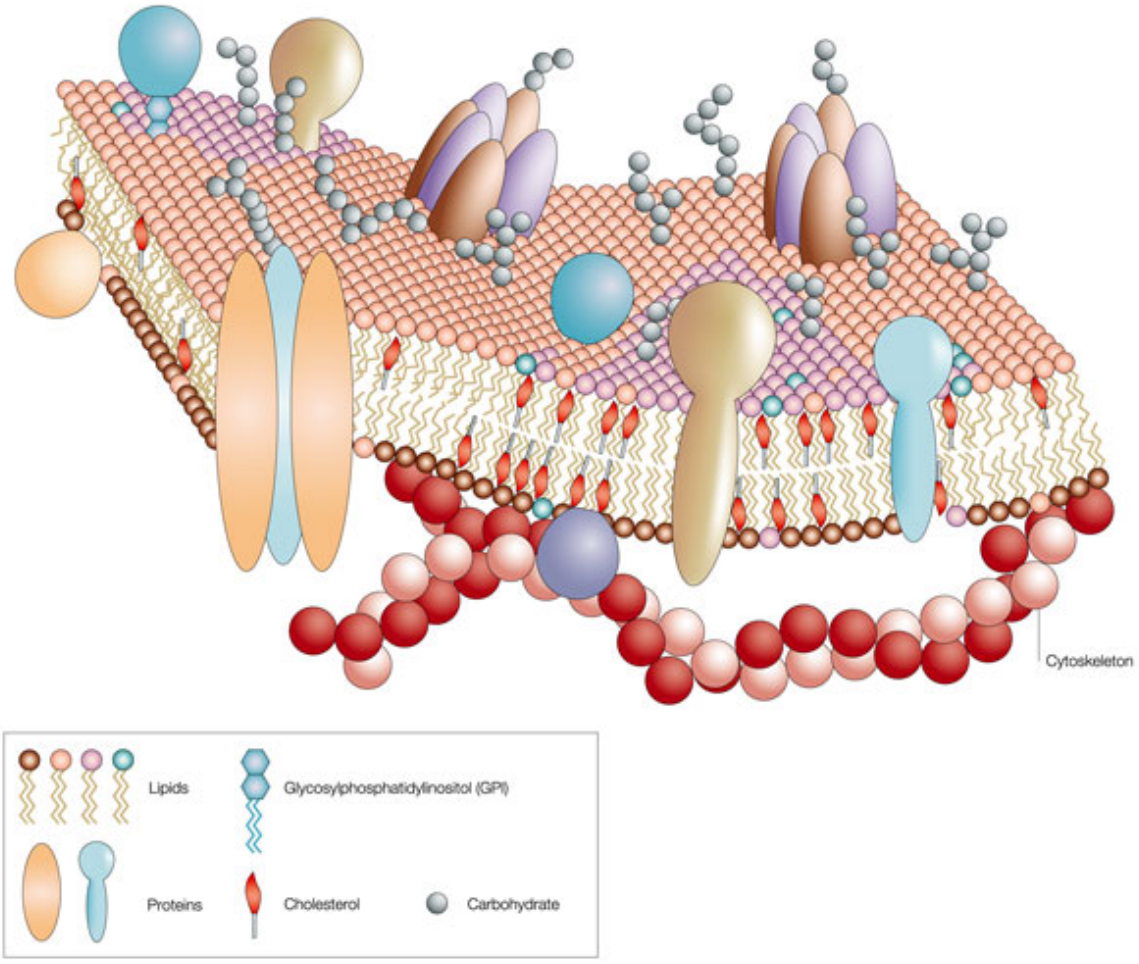


Fig 2.1 The figure illustrates the phospholipid bilayer and proteins mentioned above (http://www.nature.com/horizon/livingfrontier/background/images/membrane_f2.jpg).

Biological oxidation is of particular significance in the brain, suggest Halliwell & Gutteridge (1990) due to features such as the brain's relative glutathione deficiency as well as the fact that it is responsible for using a large proportion of the body's total oxygen requirement. Other factors such as the presence of large amounts of the above-mentioned polyunsaturated lipids, as well as high concentration of iron in some areas of the brain must also be taken into account.

In 1948, Bernhein *et al* concluded that the coloured complex produced when thiobarbituric acid is incubated with certain lipids is a result of the products of oxidation of unsaturated fatty acids.

Thus, a simple assay of the degree of lipid peroxidation involves the measure of thiobarbituric reactive species (TBARS). This test indicates the generation of hydroxyl radicals (Gutteridge & Halliwell, 1990). The most abundant molecule produced as a result of oxidative stress is malondialdehyde (Reiter et al, 1995) and is a reliable indicator of cell damage. This molecule reacts with two molecules of TBA, resulting in the production of a pale pink chromogen, the concentration of which can be easily established by UV Spectroscopy.

Aldehydes are produced during the breakdown of lipid hydroperoxides in biological systems (Esterbauer, 1982), the most abundant of which is malondialdehyde (Schaunenstein, 1997).

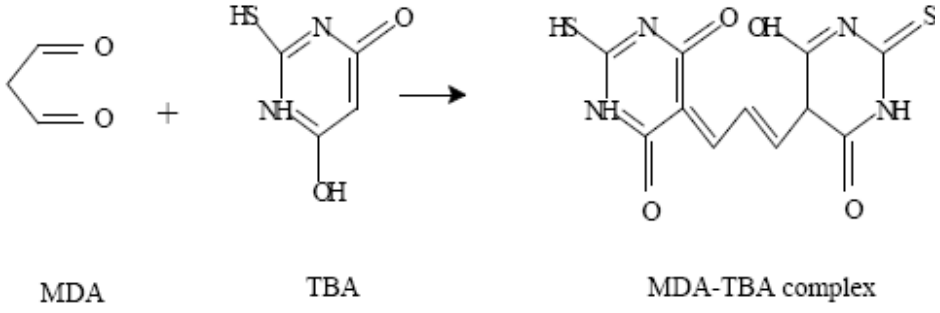


Fig 2.2 The reaction of MDA with TBA to yield a pink TBA-MDA complex (Mead *et al*, 1986).

Studies to quantify MDA were performed according to the method of Das *et al* (1990) and Sagar *et al* (1992) which was modified by Ottino and Duncan (1997). The assay involves incubating rat brain homogenate with trichloracetic acid to precipitate protein, as well as BHT to prevent additional lipid oxidation which may affect results. Centrifugation causes the precipitated protein to form a pellet so that the supernatant may be assayed with TBA and the absorbance of MDA-TBA chromogen at 532nm is used to determine the concentration from the standard curve generated using 1,1,3,3-tetramethoxypropane.

2.2 An Investigation of the Effect of QA on Rat Brain Homogenate

2.2.1 Materials & Methods

2.2.1.1 Chemicals & Reagents

DHEA was obtained from Sigma Chemicals Co, St Louis, USA. 1,1,3,3-Tetramethoxypropane (MDA) was obtained from Fluka AG, Switzerland. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and QA were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid and butan-1-ol were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. All other chemicals and reagents were obtained locally and were of the highest quality available.

2.2.1.2 Animals

Male Wistar rats weighing 280 to 300g were randomly divided into groups (n=5) and maintained as described in *Appendix A: Animal Care*.

The rats were killed by cervical dislocation and decapitated. The skull was lifted, after making an incision through the bone on either side of the parietal suture from the foramen magnum to near the orbits on both sides of the skull. Care was taken to ensure the brains were not damaged during excision. Brains were either used immediately or frozen using liquid nitrogen and stored at -70°C for later use.

Whole brain homogenate was prepared in PBS (10% w/v) at pH 7.4 and ambient temperature in order to prevent lysosomal damage of the tissue. A Teflon®-coated glass homogenizer was used.

2.2.1.3 Preparation of the Standard Curve

A series of standards (0-50 nmol/ml) was prepared using 1,1,3,3-tetramethoxypropane (MDA) and a standard curve generated by plotting the absorbance at 532nm against the molar equivalent weight of MDA in the complex assayed (see *Appendix B: Malondialdehyde Calibration Curve*). The absorbance was read using a Shimadzu UV-160A UV-visible recording spectrophotometer. Each point on the graph is the mean of triplicate readings.

2.2.1.4 The Thiobarbituric Acid Reactive Species Assay

Clean, dry test tubes were used to prepare solutions in triplicate containing 0.8ml whole brain homogenate as mentioned above, 0.1ml of 50% ethanol and 0.1ml of QA, prepared in MilliQ water (2.5mM, 5mM or 10mM) to produce final concentrations of 0.25, 0.5 and 1mM, as well as a control containing no QA.

Table 2.1 Scheme for assay of quinolinic acid induced lipid peroxidation.

Control	0.8ml homogenate	0.1ml 50% EtOH	0.1ml Water
0.25 mM	0.8ml homogenate	0.1ml 50% EtOH	0.1ml 2.5 mM QA
0.5 mM	0.8ml homogenate	0.1ml 50% EtOH	0.1ml 5mM QA
1 mM	0.8ml homogenate	0.1ml 50% EtOH	0.1ml 10mM QA

After incubating for an hour, 0.5ml BHT (0.5mg/ml in methanol) and 1ml TCA (0.15g/ml in MilliQ water) were added. The solutions were then covered and incubated for 15 minutes at 95°C. After removal from the waterbath, solutions were then centrifuged at 2000x *g* for 20 minutes. These two steps ensure that the malondiadehyde is released from the proteins to which it is bound, and prevent the adsorption of the molecule onto insoluble proteins.

Subsequently, 2ml of protein free supernatant was transferred to a clean set of test tubes and 0.5ml TBA (0.33g/ml in MilliQ water) was added, followed by extraction of the MDA-TBA complex with 2ml butan-1-ol. The solutions were centrifuged again at 2000 x *g* for 5 minutes before reading samples at 532nm using a Shimadzu UV-160A UV-visible recording spectrophotometer. Butan-1-ol was used as a blank.

2.2.1.5 Statistical Analysis

Data were analysed using ANOVA, followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

2.2.2 Results

Results are expressed as nanomoles MDA produced per milligram of wet tissue.

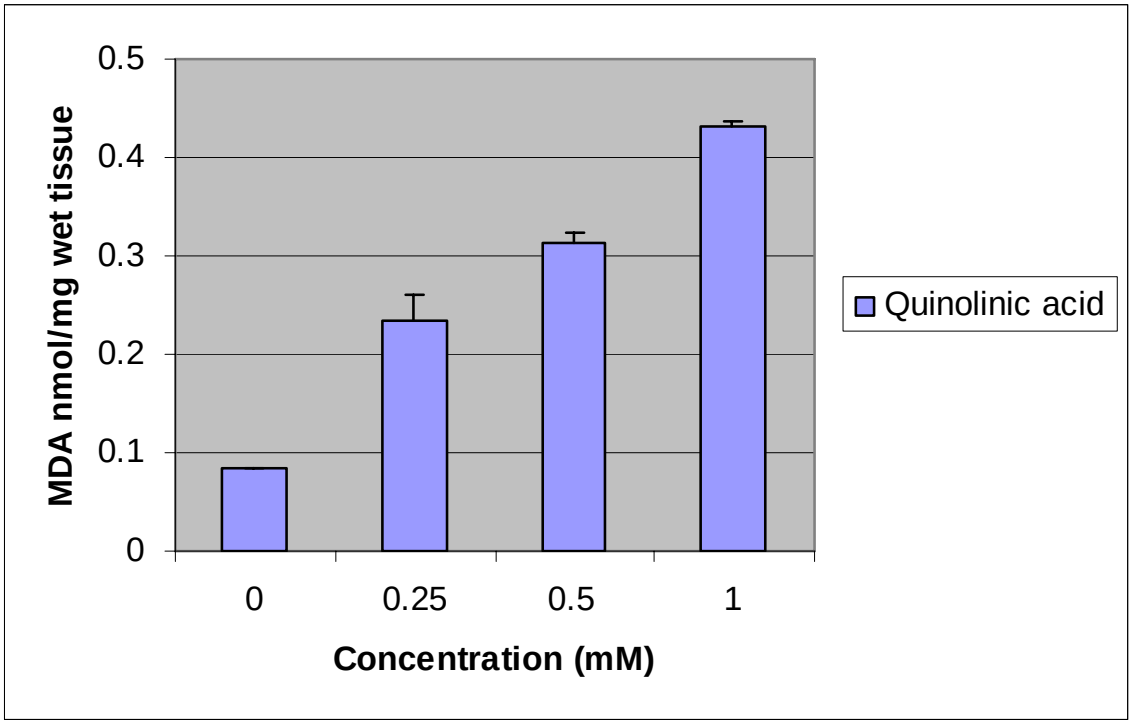


Fig 2.3 The graph shows the concentration dependent increase in induction of lipid peroxidation by quinolinic acid in rat brain homogenate. The bars represent the mean + SEM; n=5. The differences are statistically significant (Student-Newman-Keuls Multiple Range Test, $p<0.05$).

2.2.3 Discussion

These results are consistent with those obtained by other investigators, showing that QA causes a dose-dependent increase in MDA production, and therefore, lipid peroxidation. This is supported by the work of Rios and Sanatamaria (1991).

QA is an established glutamate receptor agonist which remains in the synaptic cleft and is not readily metabolised or removed, allowing it to exert its effects for a prolonged period. These include the opening of calcium channels, allowing for excess Ca^{2+} entry which in turn leads to Ca^{2+}-mediated free radical generation and ultimately cell death (Stone and Perkins, 1981).

Stipêk et al (1996) showed that the presence of an iron chelator prevents the induction of lipid peroxidation, implying that the mechanism of induction – which is not well understood – may involve these ions. QA forms a complex with ferrous ions and it is suggested that via this complex, QA may act as a pro-oxidant.

Free radical formation and oxidative stress are two mechanisms whereby QA exerts its neurotoxic effects (Pérez-Severiano *et al*, 2004)

2.3 An Investigation of the *In Vitro* Effect of DHEA on QA-induced Lipid Peroxidation

2.3.1 Materials & Methods

2.3.1.1 Chemicals & Reagents

DHEA was obtained from Sigma Chemicals Co, St Louis, USA. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and QA were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid and butanol were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. All other chemicals and reagents were obtained locally and were of the highest quality available.

2.3.1.2 Animals

Male Wistar rats weighing 280 to 300g were randomly divided into groups (n=5) and maintained as described in *Appendix A: Animal Care*. The rats were killed, the brains were removed and whole brain homogenate was prepared as detailed in Section 2.2.1.2 above.

2.3.1.3 The Thiobarbituric Acid Reactive Species Assay

The experiment was performed according to the method of Ottino and Duncan (1997) as detailed in Section 2.2.1.4 above with the following modifications: each solution had a final concentration of 1mM QA. In addition, 0.1ml DHEA (prepared in 50% ethanol) was added to give a final concentration of 0.25, 0.5 or 1mM, as well as a control containing no DHEA. The solutions were incubated at 37°C in an oscillating water bath for 1 hour and the assay continued as in Section 2.2.1.4.

Table 2.2 Scheme for determining the impact of increasing concentration of DHEA on the degree of lipid peroxidation induced by 1mM QA.

Control	0.8ml homogenate	0.1ml 50% EtOH	0.1ml Water
0.25 mM	0.8ml homogenate	0.1ml 2.5mM DHEA	0.1ml 10mM QA
0.5 mM	0.8ml homogenate	0.1ml 5mM DHEA	0.1ml 10mM QA
1 mM	0.8ml homogenate	0.1ml 10mM DHEA	0.1ml 10mM QA

2.3.1.4 Statistical Analysis

The results were analysed using a one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

2.3.2 Results

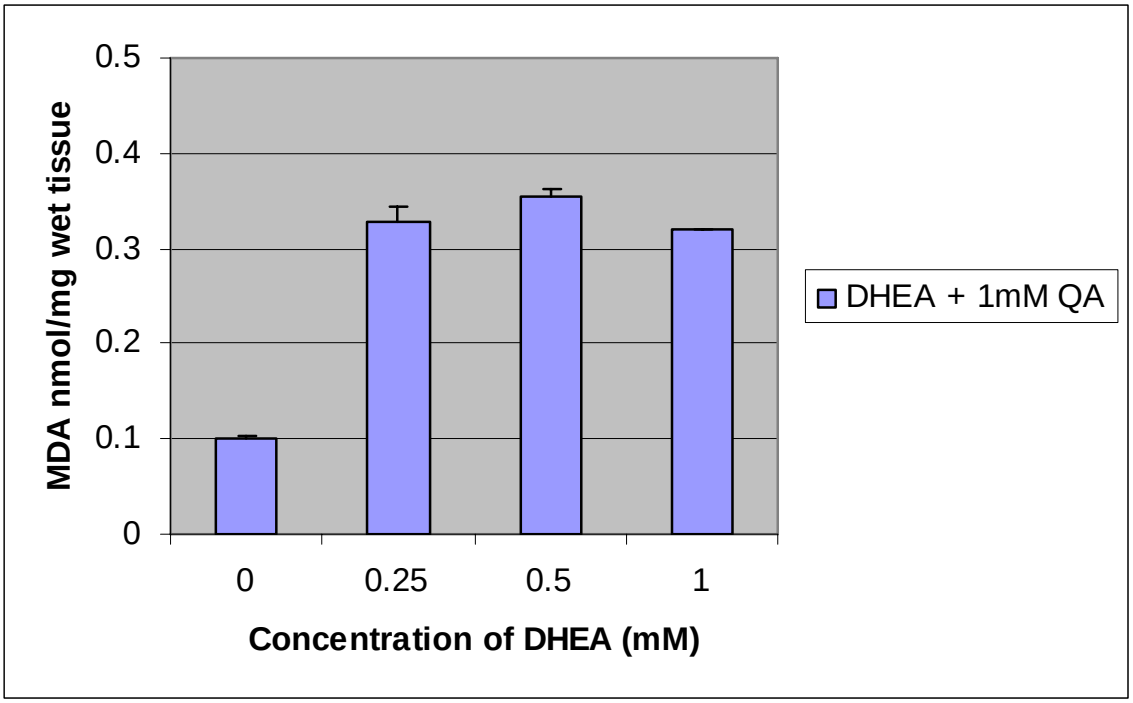


Fig 2.4 The graph represents the effect of increasing concentrations of DHEA (0-1mM) on the peroxidant effect of 1mM QA as illustrated in Fig 2.3 above. The bars represent the mean + SEM; n=5. The differences between the three concentrations of DHEA were found to be statistically insignificant (Student-Newman-Keuls).

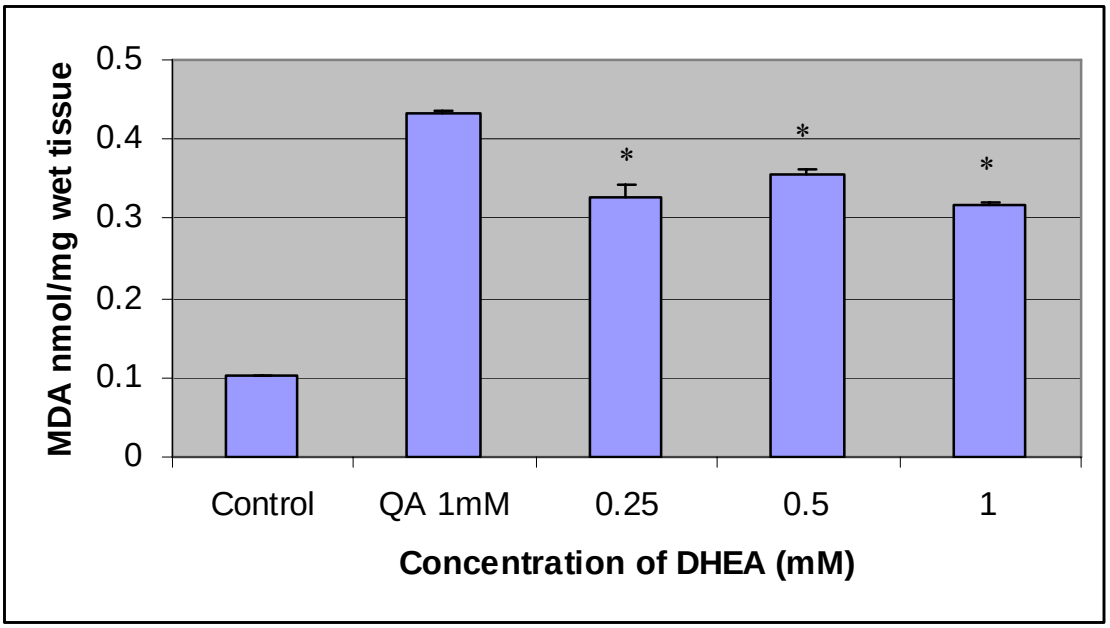


Fig 2.5 The figure shows the peroxidant effect of 1mM QA on whole rat brain homogenate, as well as the amelioration of this phenomenon by various concentrations of DHEA (0.25-1mM). The bars represent the mean + SEM; n=5. No significant difference exists between the three concentrations of DHEA. * indicates a significant decrease when compared to 1mM QA ($p<0.001$, Student-Newman-Keuls Multiple Range Test).

The reduction of TBARS at all concentrations of DHEA is statistically significant when compared to the maximum concentration of 1mM QA, while the difference in lipid peroxidation at concentrations of 0.25mM, 0.5mM and 1mM is non-significant. The amount of lipid peroxidation measured in brain homogenate incubated with 0.5mM QA is not significantly different from homogenate incubated with a combination of double the concentration of QA and 0.5mM DHEA.

2.3.3 Discussion

From the above graphs it is evident that DHEA has a favourable effect on the degree of lipid peroxidation induced by quinolinic acid. However, some lipid peroxidation occurs even when concentrations of QA and DHEA are the same, implying that the mechanism of action is not a 1:1 interaction between the two chemicals where DHEA prevents QA from inducing damage. Even when the concentration of DHEA is a quarter of that of QA, there is a significant reduction in the production of TBARS ($p<0.001$).

The protective effect does not appear to be dose-dependent at the concentrations used.

Van Rensburg and co-workers (2000) compared the free radical scavenging abilities of DHEAS to that of the efficient free radical scavenger, melatonin, and found that DHEAS reduces lipid peroxidation at both high and low concentrations.

2.4 An Investigation of the *In Vivo* Effect of DHEA on QA-induced Lipid Peroxidation in the Rat Hippocampus

DHEA has been shown to have a favourable effect on lipid peroxidation *in vitro*. However, an *in vivo-in vitro* correlation cannot be assumed. The following experiment investigates whether subcutaneously administered DHEA can protect the brain against damage caused by chemical insult to the hippocampus.

2.4.1 Materials & Methods

2.4.1.1 Chemicals & Reagents

DHEA was obtained from Sigma Chemicals Co, St Louis, USA. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and QA were purchased from Sigma Chemical Co, St. Louis, USA. Trichloroacetic acid and butanol were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. All other chemicals and reagents were obtained locally and were of the highest quality available.

2.4.1.2 Animals

Male Wistar rats weighing 280 to 300g were randomly divided into groups (n=5) and maintained as described in *Appendix A: Animal Care*.

2.4.1.3 Treatment Protocol

The rats in each group received a daily subcutaneous injection as laid out below in Table 2.3. Surgery was performed on Day 8 as indicated and daily injections were continued until Day 14. Rats were sacrificed by cervical dislocation. The rats were then decapitated and the skull lifted after making an incision through the bone on both sides of the skull, from the foramen magnum to near the orbits, taking care to ensure the brains were not damaged.

Table 2.3 Dosing regimen for lipid peroxidation studies

Group Name	Daily Subcutaneous Injection	Bilateral Intrahippocampal Injection on Day 8
Control	Vehicle	PBS
Toxin	Vehicle	120nmol Quinolinic acid
5mg/kg	DHEA in Vehicle	120nmol Quinolinic acid
10mg/kg	DHEA in Vehicle	120nmol Quinolinic acid
20mg/kg	DHEA in Vehicle	120nmol Quinolinic acid

2.4.1.4 Surgery

Surgery was performed on day 8. Rats were anaethetised by placing them individually in a dessicator lined with cotton wool soaked in diethylether. The rat was then placed in a rat brain stereotaxic frame (Stoelting, IL, USA) and level of anaethesia was maintained by placing cotton wool soaked in diethylether near the nose of the rat. The proximity of the cotton wool could be adjusted during surgery to ensure that the rat remained at the appropriate level of unconsciousness. This was determined by monitoring respiration and colour of the extremities.

After a saggital incision, the cranium was exposed and the bregma identified. Using the co-ordinates indicated below (König & Klippel, 1963), the hippocampus was located and 120nmol quinolinic acid was injected bilaterally using a Hamilton syringe with a cannula diametre of 0.3mm.

Relative to the bregma:

0.26cm laterally

0.40cm caudally

0.35cm ventrally

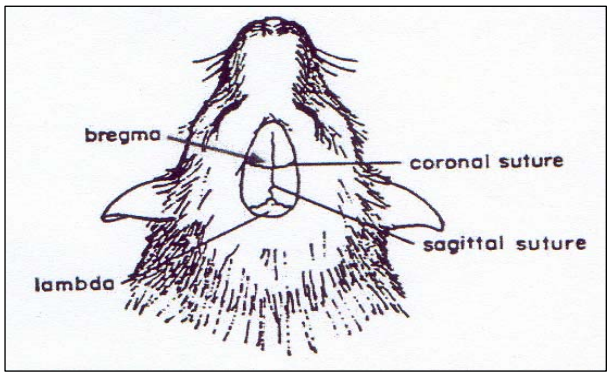


Fig 2.6 The diagram illustrates the position of the bregma, as well as the coronal and sagittal sutures (Daniels, 2002).

The solutions were administered at a rate of 1µL per minute. The cannula was left *in situ* for 2 minutes following injection to ensure diffusion away from the tip into the hippocampus and reduce possibility of spread into the injection tract.

The rat was removed from the stereotaxic frame and the incision was closed by suturing. The rat was subsequently allowed to regain consciousness while being monitored for behavioural changes due to QA-induced neurotoxicity such as seizures.

2.4.1.5 Sham-lesioned Rats

The rats in the control group were treated as detailed above in Section 2.4.1.4. However, instead of administering QA, an equal volume of PBS was injected intrahippocampally.

2.4.1.6 Isolation of the hippocampus

The hippocampi were carefully isolated and all adhering tissue removed. This was performed by following a modified method of Glowinski & Iverson (1966). After removal of the brain, the rhombencephalon was detached by a transverse section. A further transverse section at the level of the optic chiasma divided the cerebrum. The hypothalamus and striatum were subsequently dissected from the brain to access the hippocampus. The mid brain was gently separated from the remaining portion of the brain and the hippocampus was easily isolated.

2.4.1.7 The Thiobarbituric Acid Reactive Species Assay

Pooled hippocampal homogenate (10% w/v) was prepared in PBS for use in this assay. The hippocampi had already been exposed to DHEA over the past 14 days, resulting in the incubation at 37 ° C being unnecessary. Thus 0.5ml BHT (0.5mg/ml in methanol) and 1ml TCA (0.15g/ml in MilliQ water) were added directly to the freshly prepared homogenate and the assay was continued as described previously in Section 2.2.1.4.

2.4.1.8 Statistical analysis

The results were analysed using a one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

2.4.2 Results

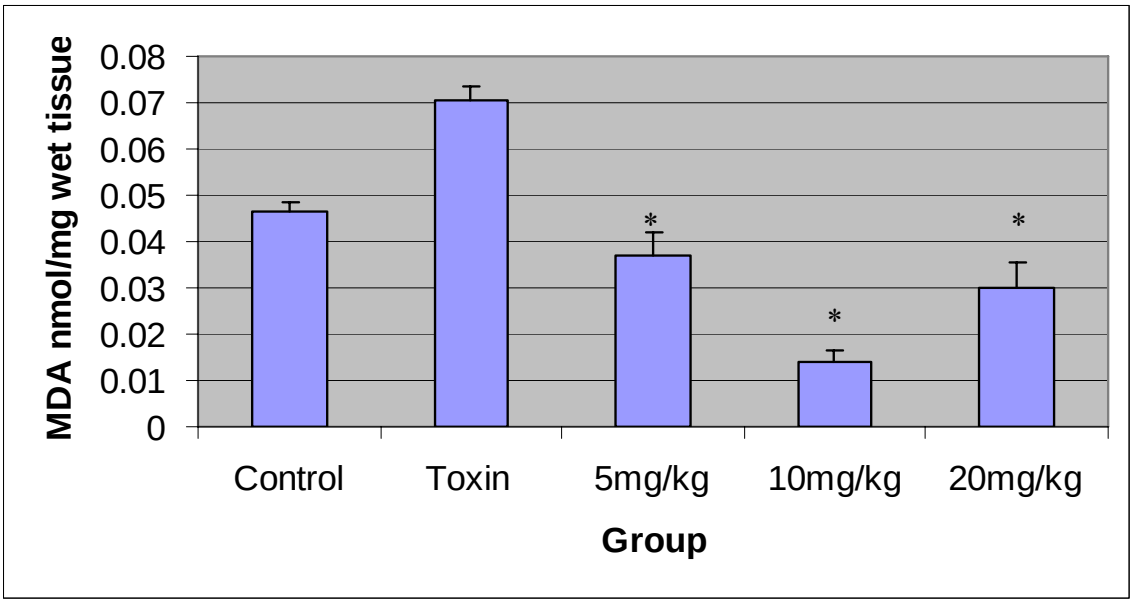


Fig 2.7 The graph demonstrates the effect of various concentrations of DHEA (5mg/kg, 10mg/kg and 20mg/kg) on intrahippocampal QA-induced lipid peroxidation where *Control* refers to sham-lesioned rats and *Toxin* refers to the group receiving a daily injection of vehicle alone. Each bar represents the mean + SEM; n=5. * represents a significant decrease relative to Toxin group ($p<0.001$, Student-Newman-Keuls Multiple Range Test)

2.4.3 Discussion

The results show a statistically significant decrease in the amount of MDA produced, indicating that there is reduced lipid peroxidation caused by quinolinic acid insult in rats treated daily with DHEA. These results are supported by the *in vitro* work outlined in Section 2.3 above where the presence of DHEA caused reduced lipid peroxidation induced by 1mM QA. QA is known to induce lipid peroxidation (Ríos & Santamaría, 1991). As with the *in vitro* study, the reduction in lipid peroxidation is not dose-dependent.

In all cases, groups treated with DHEA exhibit reduced levels of TBARS when compared with the control, indicating that DHEA doesn't merely interact with the QA and prevent it from harming the tissue, but is protective of the tissues in the hippocampus by some mechanism. This was not observed in the *in vitro* assay.

Tissue disrupted by homogenisation tends to undergo lipid peroxidation more easily than healthy, intact tissue. It is suggested that inactivation or dilution of antioxidants, as well as the release of metal ions such as iron from intracellular storage sites may explain this phenomenon (Halliwell & Gutteridge, 1989). This in turn explains the formation of the MDA-TBA complex when no QA is present as in the controls.

The results show that DHEA has the ability to oppose membrane damage *via* lipid peroxidation, suggesting that its role as a potential neuroprotectant has a basis. The potential neuroprotection suggested by the DHEA dose-dependent reduction in TBARS is supported by behavioural monitoring. This corresponds with the observed results in Section 2.4. DHEA and its derivatives have also been shown to have a modulatory effect on membrane fluidity (Morissette *et al*, 1999). Southgate *et al* (1998) showed that melatonin, a well known free radical scavenger, significantly reduces the amount of lipid peroxidation induced by QA insult. Thus the ability of a compound to reduce the degree of lipid peroxidation may indicate its value as a neuroprotectant.

The peroxidative effect of QA has already been discussed in terms of its interaction with iron and the possible interaction between DHEA and iron is further discussed in *Chapter 7: Metal Interaction Studies*.

Superoxide Anion Generation Investigation

3.1 Introduction

The biological importance and generation of the superoxide radical is discussed in *Chapter 1: Literature Review*. This may be produced due to electron leakage from the mitochondrial electron transport chain (McCord, 1985) or though the activation of certain enzymes leading to this reactive radical (Patel *et al*, 1986). In this light, a simple and reliable indicator for the detection of the superoxide anion is desirable and the nitroblue tetrazolium assay is widely regarded as such (Ottino & Duncan, 1997). In the assay, the NBT ion is converted in the presence of superoxide anions to the insoluble diformazan, the concentration of which can be determined at 560nm.

Cyanide is used to induce the generation of superoxide anions since QA, the toxin previously used in *Chapter 2: Lipid Peroxidation Investigation*, has been demonstrated not to significantly alter the production of this free radical at these concentrations. This finding holds true for other glutamate agonists previously investigated (Southgate, 1998). An investigation of cyanide-induced histotoxic hypoxia by Johnson *et al* (1986) showed that KCN induces a rise in brain calcium levels. This can result in the elevation of calcium in the cytosol and is related to free radical generation. Cyanide has been demonstrated to inhibit anti-oxidant enzymes, which may add to its neurotoxicity (Ardelt *et al*, 1989).

The chapter investigates the effect of incubating whole rat brain homogenate with increasing concentrations of cyanide, and the resultant effect on superoxide anion production. The effect of DHEA on superoxide generation is also examined.

3.2 An Investigation of the Effect of Cyanide on Rat Brain Homogenate

3.2.1 Materials & Methods

3.2.1.1 Chemicals & Reagents

Nitro-blue tetrazolium (NBT), Nitro-blue diformazan (NBD), potassium cyanide (KCN) and bovine serum albumin (BSA) were purchased from the Sigma Chemical Company (USA). Glacial acetic acid was purchased from Holpro Chemical Company (South Africa). Folin's reagent was purchased from Saarchem (PTY) LTD, Krugersdorp, South Africa. All other chemicals and reagents were obtained locally and were of the highest quality available.

A 0.1% NBT solution was made by dissolving the NBT in ethanol before making up to the required volume with Milli-Q water. The final ethanol concentration in the incubation flasks was less than 1.5%.

3.2.1.2 Animals

Male Wistar rats weighing 280 to 300g were randomly divided into groups (n=5) and maintained as described in *Appendix A: Animal Care*. The rats were killed by cervical dislocation and decapitated. The skull was lifted, after making an incision through the bone on either side of the parietal suture from the foramen magnum to near the orbits on both sides of the skull. Care was taken to ensure the brains were not damaged during excision. Brains were either used immediately or frozen using liquid nitrogen and stored at -70°C for later use.

Whole brain homogenate was prepared in PBS (10% w/v) at pH 7.4 and ambient temperature in order to prevent lysosomal damage of the tissue. A Teflon®-coated glass homogenizer was used.

3.2.1.3 Preparation of the Standard Curve

A series of standards (0-400µmol/L) was prepared using NBD and a standard curve generated by plotting the absorbance at 560nm against the molar equivalent weight of NBD in the complex assayed (see Appendix C: Diformazan Calibration Curve). The absorbance at 560nm was read using a Shimadzu UV-160A UV-visible recording spectrophotometer. Each point on the graph is the mean of triplicate readings.

3.2.1.4 The Nitro Blue Tetrazolium Assay

The method is performed according to the methods of Sagar *et al* (1992) and Das *et al* (1996), as modified by Ottino and Duncan (1997). Clean, dry test tubes were used to prepare solutions in triplicate containing whole brain homogenate as mentioned above and various concentrations of KCN (0-1mM). The solutions were incubated with 0.4ml 0.1% NBT for 1 hour in an oscillating water bath set to 37°C. After incubation, the samples were centrifuged at 2000g and the remaining pellet was resuspended in 2ml glacial acetic acid. The absorbance of the glacial acetic acid fraction was measured at 560nm and the concentration of diformazan in µmoles was determined using the standard curve generated in 3.2.1.3.

Protein content of homogenate was determined using a modified method of Lowry *et al* (1951). Clean, dry test tubes were used to prepare a solution containing 6ml of alkaline copper reagent (shown in Table 3.1 below) and 1ml homogenate and allowed to stand at room temperature for ten minutes. Subsequently, 0.3ml Folin's reagent was added and tubes were vortexed and allowed to stand at room temperature in the dark for thirty minutes. The absorbance was read at 500nm using a Shimadzu UV-160A UV-visible recording spectrophotometer and results were compared to the standard curve generated using 0-300 µg/ml and 1ml BSA *in lieu* of homogenate (see *Appendix D: Protein Assay Calibration Curve*).

Table 3.1 The components of the alkaline copper reagent

Reagent (concentration)	Volume
Copper sulphate (1%)	1ml
Sodium Tartrate (2%)	1ml
Sodium Carbonate (2%)	2ml

3.2.1.5 Statistical Analysis

Data were analysed using ANOVA, followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

3.2.2 Results

Results are expressed as µmol diformazan produced per mg protein.

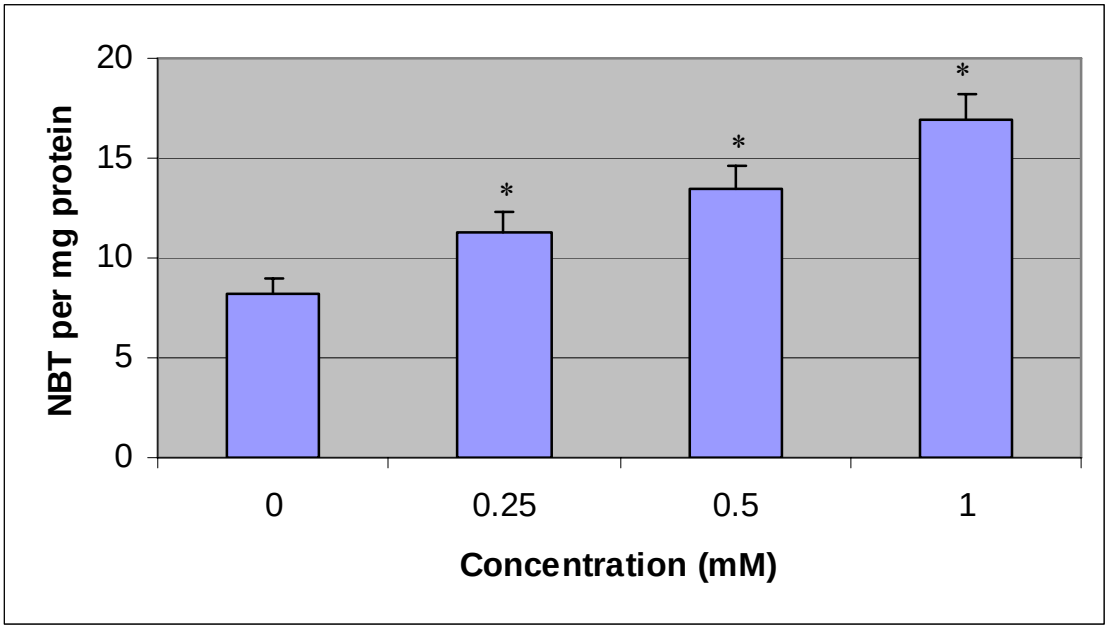


Fig 3.1 The graph shows the effect of varying concentrations of KCN (0.25-1Mm) on superoxide anion generation in whole rat brain homogenate. The bars represent the mean + SEM; n=5. * indicates a significant difference between the three concentrations used (p>0.001, Student-Newman-Keuls Multiple Range Test)

3.2.3 Discussion

The results show a significant increase in superoxide anion generation in a concentration dependent manner in the presence of cyanide when compared to the control. Thus, the toxin does exert toxic effects through increased free radical generation in this investigation and as reported previously (Lambat *et al,* 2000). The increase of 1mM KCN is approximately 80% relative to the control and was selected for the subsequent investigation since it caused the greatest superoxide anion generation.

3.3 An Investigation of the *In Vitro* Effect of DHEA on Cyanide-induced Superoxide Anion Generation

3.3.1 Materials & Methods

3.3.1.1 Chemicals & Reagents

Nitro-blue tetrazolium (NBT), Nitro-blue diformazan (NBD), potassium cyanide (KCN), bovine serum albumin (BSA) and DHEA were purchased from the Sigma Chemical Company (USA). Glacial acetic acid was purchased from Holpro Chemical Company (South Africa). Folin's reagent was purchased from Saarchem (PTY) LTD, Krugersdorp, South Africa. All other chemicals and reagents were obtained locally and were of the highest quality available.

A 0.1% NBT solution was made by dissolving NBT in ethanol before making up to the required volume with Milli-Q water. The final ethanol concentration in the incubation tubes was below 1.5%.

3.3.1.2 Animals

Male Wistar rats weighing 280 to 300g were randomly divided into groups (n=5) and maintained as described in *Appendix A: Animal Care*. The rats were killed and brains were removed and whole brain homogenate was prepared as detailed in Section 3.2.1.2 above.

3.3.1.3 The Nitro-blue Tetrazolium Assay

Clean, dry test tubes were used to prepare solutions in triplicate containing whole brain homogenate as mentioned above, 1mM KCN and various concentration of DHEA (0-1mM) The solutions were incubated with 0.4ml 0.1% NBT for 1 hour in an oscillating water bath set to 37°C. The assay was then continued as outlined in Section 3.2.1.4 above.

3.3.1.4 Statistical Analysis

Data were analysed using ANOVA, followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (Zar, 1974).

3.3.2 Results

Results are expressed as µmol diformazan produced per mg protein.

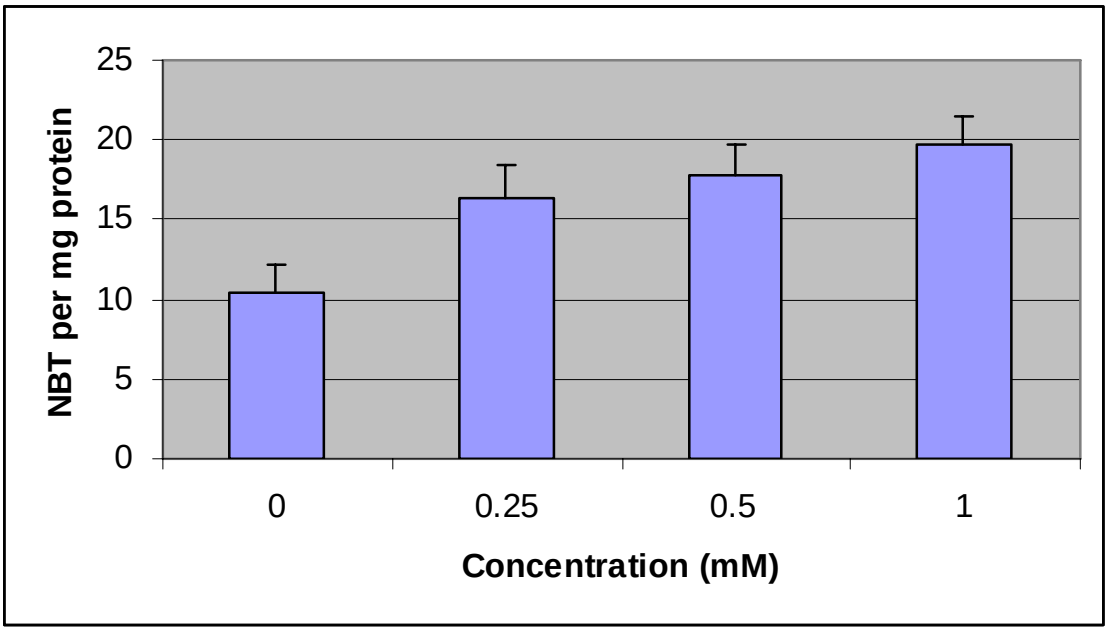


Fig 3.2 The graph shows the effect of varying concentrations of DHEA (0.25-1Mm) on KCN-induced superoxide anion generation in whole rat brain homogenate. The bars represent the mean + SEM; n=5. No significant difference exists between the three concentrations of DHEA (Student-Newman-Keuls Multiple Range Test).

There is no significant difference in KCN-iduced superoxide generation in the presence or absence of DHEA.

3.3.3 Discussion

DHEA was unable to reduce the concentration dependent induction of superoxide radicals by KCN at the concentrations used. DHEA does not interact with KCN at these concentrations, nor does it scavenge the superoxide radical. It therefore cannot be deduced that DHEA is a potent inhibitor of superoxide anion generation by cyanide in the brain, and must mediate its neuroprotective effects through another mechanism. Further studies on the electron transport chain may be of value to elucidate other effects of DHEA, as well as an investigation using higher concentrations of DHEA as other researchers have shown that DHEA may act as a pro- or an anti-oxidant, depending on its concentration (Mastrocola *et al*, 2003). Additionally, KCN has been shown to elevate calcium levels as mentioned above, yet DHEA has been demonstrated to play a role in calcium homeostasis (Watter & Dorsa, 1998) and a further investigation of this may be beneficial.

Apoptosis

4.1 Introduction

Programmed cell death is a term used almost interchangeably with apoptosis. More correctly, apoptosis is a form of programmed cell death, and not cell death itself (Schwartz *et al*, 1993). The mechanism of this particular type of cell death is explained in *Chapter 1: Literature Review*.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling is more commonly referred to as TUNEL and is the most frequently used method for the identification of apoptosis. Apoptosis may be identified by the apoptotic cell morphotype described by Kerr and colleagues (1972), though Negoescu *et al* (1997) maintains that the TUNEL system is more rapid and not subject to the variation in morphology due to fixation processes.

Fundamentally, Proteinase K is applied to slices of tissue to facilitate access to the nuclear targets. The TUNEL labeling mixture is subsequently added and interacts with breaks in DNA by labeling the 3'-OH termini.

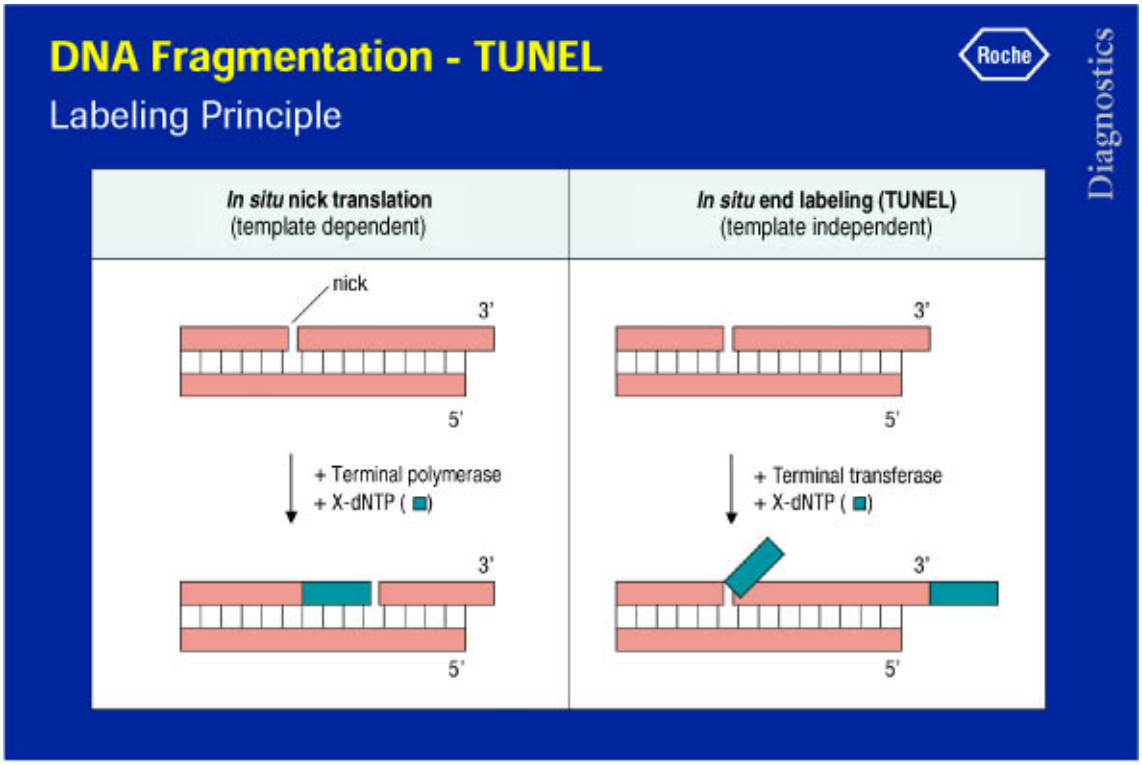


Fig 4.1 The diagram illustrates the labeling principle used in TUNEL staining. The free 3'-OH termini interact with terminal transferase (TdT), causing labelled nucleotides to be

attached (http://www.roche-applied-science.com/fst/apoptosis.htm?/sis/apoptosis/products/insitu_celldeath_detection.htm).

4.2 Materials & Methods

4.2.1 Chemicals & Reagents

The *In situ* Cell Death Detection Kit, POD, DNase and Proteinase K were purchased from Roche Diagnostics, Mannhein, Germany. DHEA and Aminopropyltriethoxysilane (APES) were purchased from Sigma Chemicals Co, St Louis, MO, USA. Shandon Histoplast Wax was purchased from Lasec, South Africa. DHEA was prepared in 10% ethanol in ethyl oleate (vehicle) and QA was dissolved in PBS.

4.2.2 Animals

Animals were maintained as described in *Appendix A: Animal Care*. Animals were subjected to the same treatment regimen as described in Section 2.4.1.3. To summarise, the dosing regimen is provided in Table 4.1.

Table 4.1 Dosing regimen for apoptotic cell death detection.

Group Name	Daily Subcutaneous Injection	Bilateral Intrahippocampal Injection on Day 8
Control	Vehicle	PBS
Toxin	Vehicle	120nmol Quinolinic acid
5mg/kg	DHEA in Vehicle	120nmol Quinolinic acid
10mg/kg	DHEA in Vehicle	120nmol Quinolinic acid
20mg/kg	DHEA in Vehicle	120nmol Quinolinic acid

4.2.3 Histological techniques for apoptosis detection

4.2.3.1 Brain fixation

After treatment, rats were sacrificed by cervical dislocation as laid out in *Chapter 2: Lipid Peroxidation Investigation*. The brains were carefully removed and placed in Davidson's fixative solution for 48 hours to prevent autolysis and bacterial destruction of the morphological

characteristics of the tissue. This is accomplished by various stabilizing or cross linking agents such as formalin, methanol and Boiun's reagent (Negoescu *et al*, 1998). No universal fixative exists; rather one must be selected based on planned use of fixed tissue as well as economic factors. The type and fixation of a tissue influence staining. Negoescu *et al* (1997) demonstrated that formalin produced favourable results with TUNEL staining compared with other fixatives such as methanol. Fixation should occur rapidly to reduce manipulation which may result in histological artifacts. Davidson's fixative acts quickly, producing good nuclear detail with minimal formalin pigment (Moore *et al*, 1953).

The brains were then transferred to 70% ethanol for storage until use.

Table 4.2 Davidson's Fixing Reagent

Volume (ml)	Reagent	Duration
335	Water	48 hours
330	Ethanol	
220	Formaldehyde	
115	Glacial Acetic Acid	

4.2.3.2 Dehydration, Clearing and Waxing Process

Before embedding of brains in wax can take place, the tissue to be embedded must be dehydrated. This is accomplished by immersing brains in increasingly concentrated alcohol solutions, followed by organic solvents to ensure all water is displaced from the tissue. The protocol, a modified method of Geiger (1997), is laid out in Table 4.3 below. The graded ethanol solutions remove moisture from the tissue and the clearing steps replace ethanol with chloroform. The wax is then allowed to penetrate the tissue and eliminate the chloroform. Vacuuming of the wax in the waxing step (Step 11 in Table 4.3 below) ensures that the wax penetrates the tissue and that no air bubbles are present, which may adversely affect the slicing of the tissue.

The process is more complex than used for other histology protocols since the TUNEL technique is more sensitive, and all reagents which may affect the fluorescence must be avoided. Prior to dehydration, a 2mm wide section of the brain was removed to exclude the site of physical damage caused by the needle during surgery. This is identifiable by a slight dimpling of the surface of the brain.

Table 4.3 The table indicates the solutions utilised, as well as the appropriate duration of immersion, for embedding brains in paraffin wax for apoptosis detection.

Step		Processing Agent	Repeat X Time (Hrs)
1	Dehydration	50% Ethanol	1 x 2
2		70% Ethanol	1 x 2
3		80% Ethanol	1 x 2
4		90% Ethanol	1 x 2
5		96% Ethanol	2 x 2
6		Absolute Ethanol	3 x 2
7	Clearing	Absolute Ethanol: Chloroform	1 x 2
8		Chloroform	1 x 2
9		Xylene: Chloroform @ 60°C	1 x 1
10	Waxing	Melted Paraffin Wax (57-58°C mp) @ 60°C	1 x 1
11		Vacuum @ 60°C	15min
12		Melted Paraffin Wax (57-58°C mp) @ 60°C	2 x 1
13	Embedding	In molten wax @ 45°C	Overnight

4.2.3.3 Embedding

The embedded sections of brain were affixed to wooden blocks and the blocks of wax were shaped using a hot blade so that the base of the wax block formed a rhombus, with the two converging sides leaving 2mm of wax surrounding the section of brain. This is graphically represented below in Fig 4.3. Tissues may then be stored indefinitely without visible influence on the quality of TUNEL (Geiger, 1997).

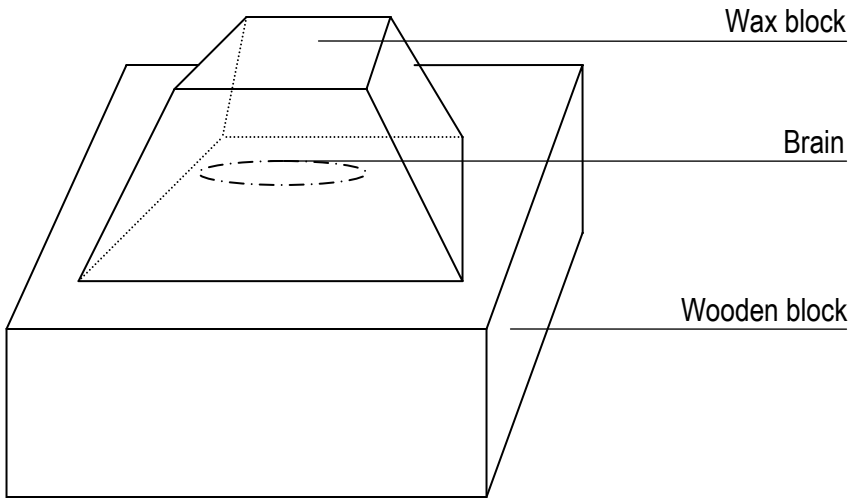


Fig 4.2 Stylistic representation of a section of brain embedded in wax and mounted on a block before sectioning.

4.2.3.4 Treating Slides

Sectons were mounted on slides coated with APES (aminopropyl triethoxysilane). Poly-L-lysine may also be used, but APES has been demonstrated to be superior in preventing tissue detachment from glass (Ben-sasson *et al*, 1995). The selection of appropriate adhesive is vital, since the procedure for TUNEL staining involves multiple washing steps and the loss of tissue is a possibility with an unsuitable adhesive.

Slides were coated with APES two days prior to use according to the method of Herrington and McGee (1992). The slides were immersed in 2% Decon 90 (prepared in distilled water, warmed to 60°C) for 30 minutes, then rinsed twice in distilled water and twice in acetone. Slides were then air dried at 37°C. Immersion in 2% APES in acetone for 30 minutes was followed by rinsing twice in acetone and then finally distilled water. The slides were air dried at 37°C once again and stored in a cool, dry place.

4.2.3.5 Sectioning

Sections were cut from the mounted block using a rotary microtome. A microtome is a specialised piece of equipment consisting of an extremely sharp blade held in position and a chuck which is used to hold the block of wax upon which the tissue is mounted. An oscillating mechanism is then utilised to move the chuck up vertically, moving it closer to the blade by a fixed distance with each oscillation (Hodgson, 1992). The microtome was set to slice sections of 5μm thickness. The shape of the wax block ensured that successive slices would form a ribbon of wax.

The block was sliced and sections discarded until the hippocampus was reached. When a ribbon of sections containing hippocampal slices was produced, the ribbon was transferred to a water bath set at 40°C using an artist's fine paintbrush. The warm water allows the wax ribbon to flatten out, which facilitates the transfer to slides.

Once ribbons were sufficiently flattened, sections were transferred to slides previously prepared in Section 4.2.3.4. The slides were placed on a warming tray to allow water to evapourate, and then placed in an oven set at 45°C overnight to allow the tissue to adhere properly to the slide.

4.2.3.6 Deparaffinising Sections

Since the TUNEL stain is aqueous, slices must be dewaxed and rehydrated before the reaction can proceed. This is accomplished first by heat, then immersion of slides in xylene, followed by ethanol as detailed below in Table 4.4.

Table 4.4 Dewaxing and rehydration protocol for brain tissue sections.

Step		Processing Agent	Repeat X Time (min)
1	Dewaxing	Heating at 60°C	20
2		Xylene	2 X 5
3	Rehydrating	Absolute Ethanol	2 X 3
4		95% Ethanol	1 X 3
5		90% Ethanol	1 X 3
6		80% Ethanol	1 X 3
7		70% Ethanol	1 X 3

4.2.3.7 Detection of Apoptotic Cells using TUNEL

Apoptotic cells were identified by treating sections of brain with the Roche *In Situ* Cell Detection Kit, according to instructions found in the manual at www.roche-applied-science.com/pack-insert/1684817a.pdf. The Roche kit is highly sensitive, produces the best signal-to-background ratio and is user-friendly when compared with existing lab protocols and other detection kits (Negoescu *et al*, 1998).

Slides previously deparaffinised were rinsed twice in PBS. In order to ensure access to DNA in the nucleus, Proteinase K (20 µg/ml in 10mM Tris-HCl, pH 7.4-8) was added to tissue and slides were incubated at 37°C in a humidified chamber for fifteen minutes. Proteinase K accomplishes this by digesting cross-linked proteins which increases cell permeability. This concentration is optimised to prevent tissue damage and reduce the possibility of non-specific staining. During this incubation and all subsequent incubations Parafilm® was placed over slides to prevent dehydration. This step also ensures that the reaction mixture is evenly distributed over the entire section. The reaction was terminated by washing thrice in PBS.

Positive and negative controls were prepared due to differences in TUNEL detection between experimental runs. One positive control was prepared by removing a slide pre-treated with Proteinase K as above and treating it with DNase I (3000U/ml in 50mM Tris-HCl, pH 7.4, containing 1mg/ml BSA) and incubating for ten minutes at 25°C, then washing thoroughly with PBS. The DNAse I is an endonuclease (Minnock *et* al, 1999) which hydrolyses sites adjacent to pyrimidine, resulting in intensive labelling of all nuclei. It was prepared immediately before use as thawing from frozen results in inactivation of the compound.

The TUNEL reaction mixture was prepared during the incubation time of the step described above and maintained in a dark environment since mixing of the components results in a light sensitive solution. The reaction mixture was kept on ice during labelling. Firstly, 100µl of the labelling solution from Vial 2 (a nucleotide mixture in reaction buffer) was removed to use on the negative controls. The balance of Vial 2 was subsequently combined with the enzyme solution in Vial 1. This enzyme solution is terminal deoxynucleotidyl transferase (Tdt) from calf thymus (recombinant in *E. coli*) in a storage buffer.

4.2.3.8 TUNEL Labelling

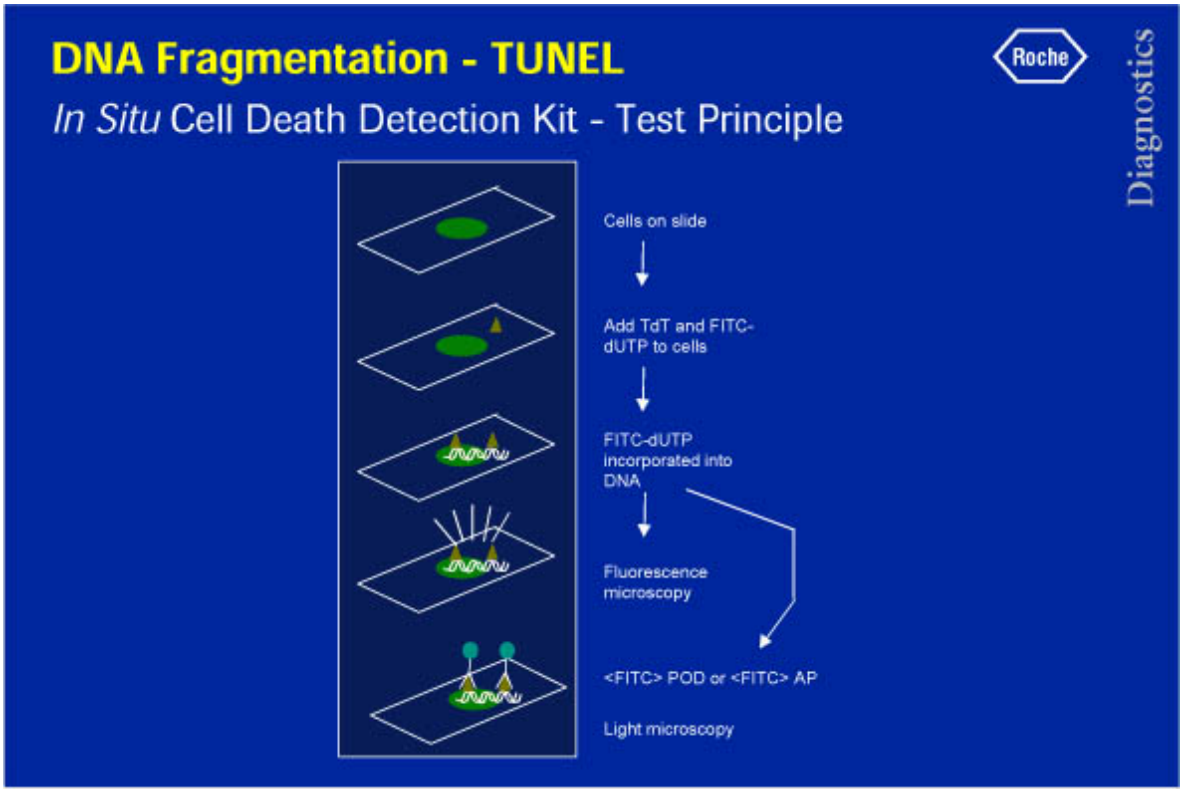


Fig 4.3 The diagram summarises the steps explained in Sections 4.2.3.5 to 4.2.3.9. The diagram demonstrates an extra step whereby the fluorescence can be converted using a substrate to visualise apoptotic bodies with light microscopy (http://www.roche-applied-science.com/fst/apoptosis.htm?/sis/apoptosis/products/insitu_celldeath_detection.htm).

TUNEL reaction mixture (50µl) was added to each test section, as well as to the positive control slides which had been pre-treated in Section 4.2.3.7 with DNase I. The negative control received 50µl of pure labelling solution. All slides were placed in a humidified chamber at 37°C for one hour for the end-labelling to occur. The temperature may not increase above this value as temperatures over 40°C inactivate the enzyme (Geiger, 1997). After one hour, slides were immersed in PBS and then washed thrice in PBS to ensure termination of the reaction.

4.2.3.9 Microscopy

Prior to viewing slides, coverslips must be placed over the tissue to improve visibility and prevent damage. This was accomplished by first adding sufficient SHUR/MOUNT ™, an aqueous mountant, to each slide and then gently lowering a coverslip into place. Photomicrographs were immediately taken using an Olympus digital camera attached to a fluorescent detector at 200X magnification. The fluorescence of the apoptotic cells is detected in the range 515-565nm which is why the photomicrographs appear green. Sections were protected from light during viewing and photomicroscopy.

4.3 Results

The figures below are photomicrographs of brain tissue from rats treated as outlined above in Section 4.2.2, and treated with the TUNEL stain.

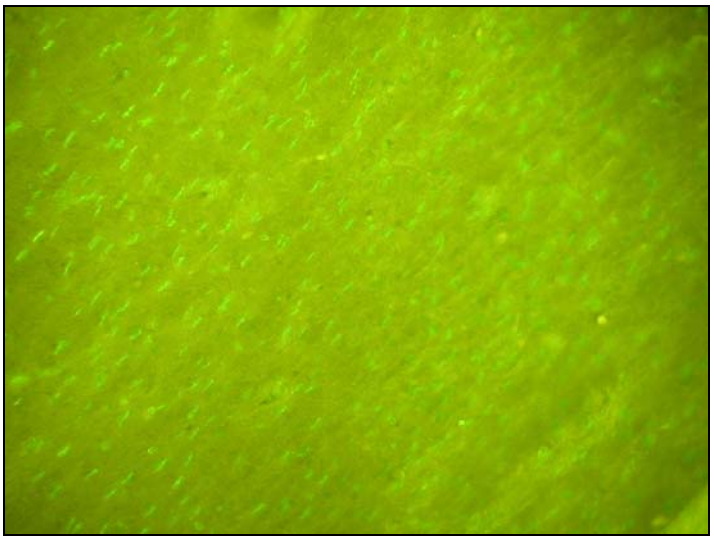


Fig 4.4 The photomicrograph is the positive control. Hippocampal tissue was treated with DNAse I prior to TUNEL staining, which induced extensive DNA fragmentation and thus widespread fluorescent labeling (magnification X 200).

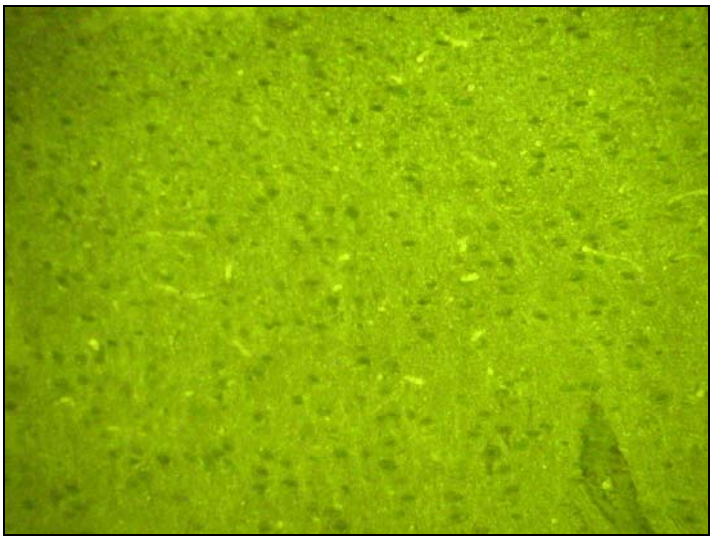


Fig 4.5 The photomicrograph of hippocampal tissue of rat receiving daily subcutaneous injections of the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained PBS alone (magnification X 200).

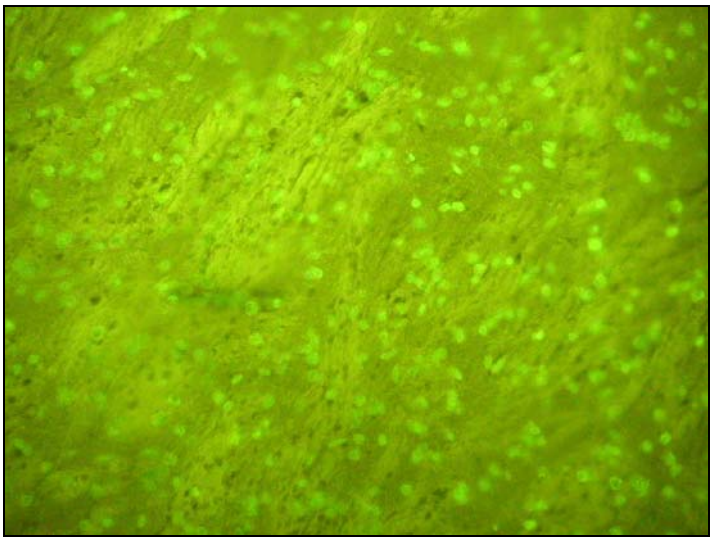


Fig 4.6 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 200).

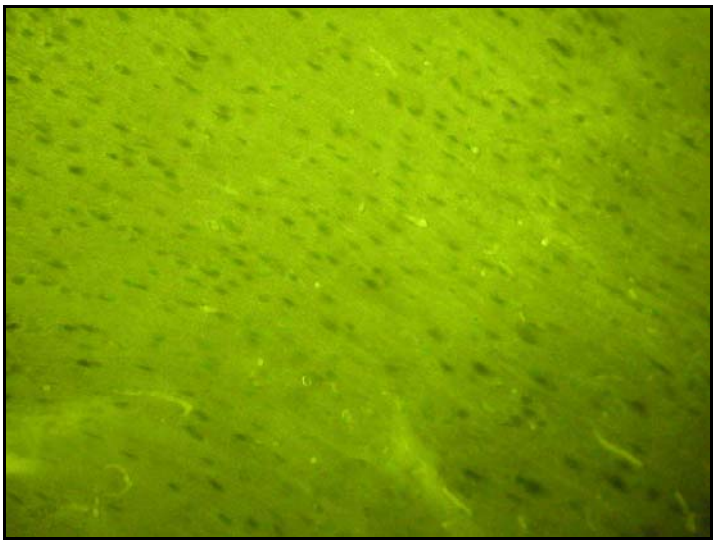


Fig 4.7 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 5mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 200).

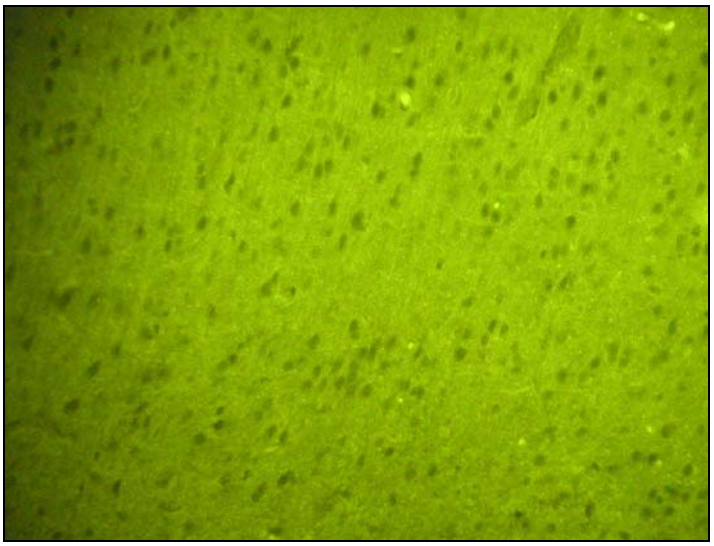


Fig 4.8 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 10mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 200).

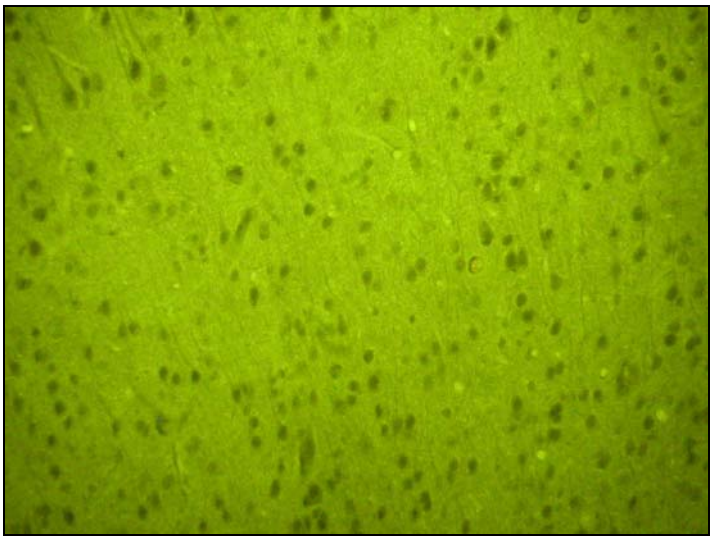


Fig 4.9 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 20mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 200).

The following images are from brain tissue of rats which received 5mg/kg, 10mg/kg and 20mg/kg DHEA subcutaneously daily as above in Section 4.2.2. These rats were sham-operated and were not exposed to neurochemical insult intrahippocampally.

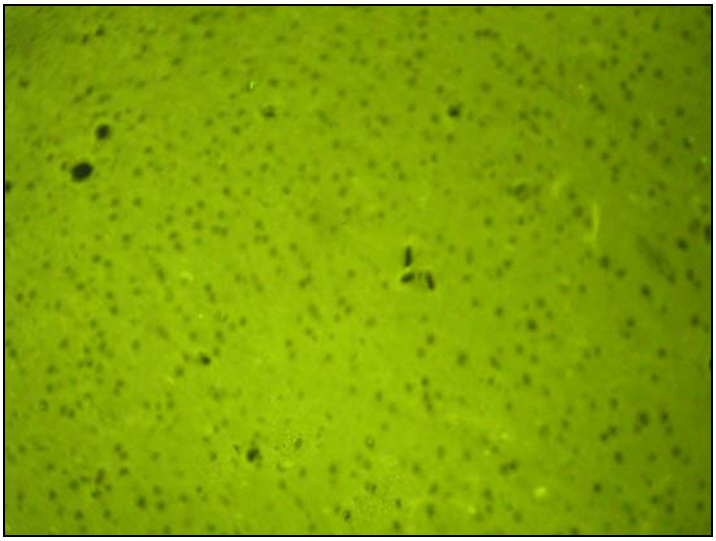


Fig 4.10 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 5mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained PBS alone (magnification X 200).

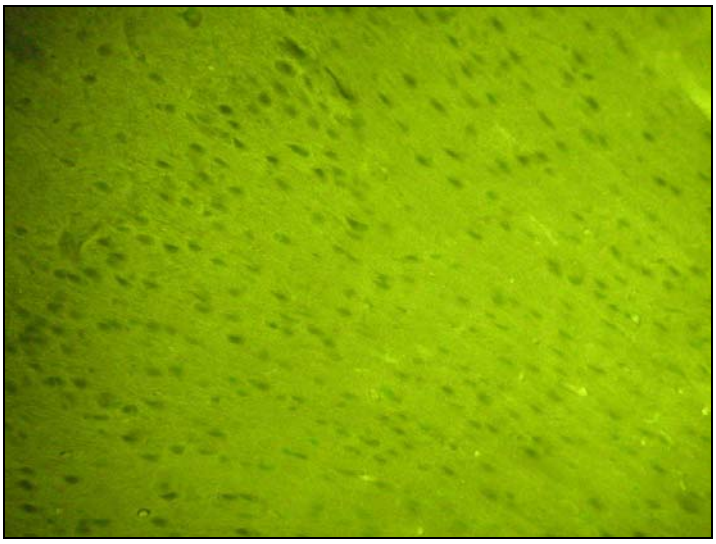


Fig 4.11 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 10mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained PBS alone (magnification X 200).

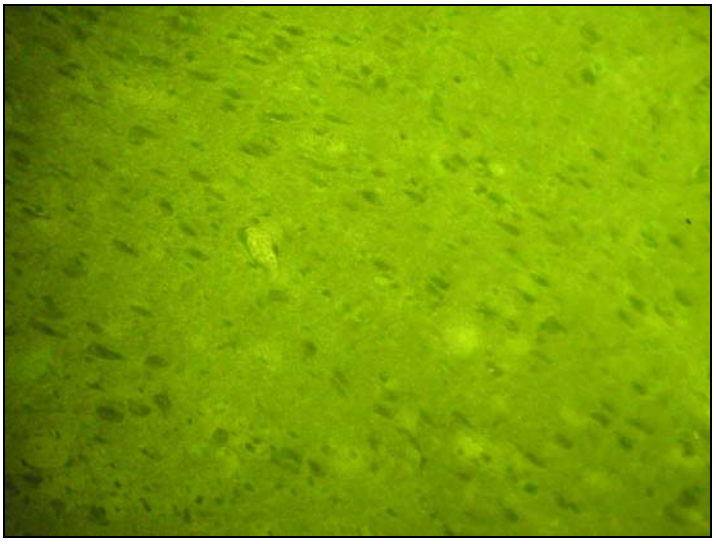


Fig 4.12 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 20mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained PBS alone (magnification X 200).

4.4 Discussion

DNase I controls confirm that staining on the experimental slides is in fact positive staining for apoptosis and not artifact. DNase I results in intensive labeling of all nuclei throughout the tissue section.

It is evident from the images above that there is reduced fluorescence – and therefore reduced evidence of apoptosis – in all rats treated with DHEA. Even the lowest concentration of DHEA (5mg/kg) showed a remarkable decrease in the degree of apoptosis caused by intrahippocampal chemical insult by the neurotoxin QA. QA has been shown to induce apoptosis (Cammer, 2001).

The control group shows normal brain tissue with scattered fluorescent objects indicating several apoptotic cells as expected. Fig 4.6 is taken from the toxin group which received intrahippocampal QA injections. Fluorescence – and therefore apoptotic cell death – is widespread throughout the tissue due to the presence of the neurotoxin.

DHEA itself does not induce apoptosis, due to the lack of neurons which appear to be stained green in Figs 4.9-4.11. This is supported by Kaper and Mueller (1999) who demonstrated that DHEA alone has no effect on spontaneous apoptosis. The figures demonstrate that at these concentrations, DHEA does not exert a pro-oxidant effect. Mastracola *et* al (2003) showed that DHEA may act as pro-oxidant at high tissue concentrations, although many texts show that DHEA has neuroprotective properties (Bastianetto *et al*, 1998; Kimonides *et al*, 1997).

The photomicrographs show that the neurosteroid protects against QA-induced apoptosis. Slices of brain tissue from rats receiving each of the three doses of DHEA (5mg/kg, 10mg/kg and 20mg/kg) show a marked reduction in fluorescence, confirming that DHEA at all concentrations has protected the neural tissue from chemical injury by the neurotoxin.

There is no visible difference between tissue from control brains and brains subjected to QA and various concentrations of DHEA, suggesting that DHEA prevents the induction of apoptosis by QA, and not merely reducing it.

These results are supported by Zhang *et* al (2002) who showed that DHEA suppresses apoptosis in cell culture by activating the serine-threonine protein kinase Akt. These authors also noted that the sulphate ester inhibits the levels of Akt, increasing apoptosis. The authors suggest that the equilibrium between DHEA and DHEAS regulates the survival of neural precursors during development of the CNS through this Akt signaling pathway (Zhang *et al*, 2002). DHEA(S) was shown to decrease staurosporine-induced apoptosis in human peripheral blood lymphocytes (Takahashi *et al*, 2004). Additionally, the authors demonstrated it was not mediated via androgen or oestrogen receptors. DHEA has been demonstrated to markedly reduce cyproterone acetate-induced apoptosis in rat hepatocytes (Kasper & Mueller, 1999).

It can be observed from the above that apoptosis is reduced relative to the control section in the brains of rats treated with DHEA before a chemical insult to the hippocampus.

Necrosis results in clusters of neighbouring cells. This is associated with the inflammatory process as immune cells infiltrate the area and disrupt the tissue. Apoptotic cells, however, are scattered or display a distinct pattern of distribution.

Histology

5.1 Introduction

Although assays such as those for lipid peroxidation and superoxide anion generation are important for determining molecular conditions, there is value in observing the influence of DHEA and quinolinic acid at various concentrations on the morphology of cells. The preceding chapter has already demonstrated that DHEA ameliorates QA-induced apoptotic cell death in the rat brain and this chapter will investigate whether DHEA administration results in any change to the morphology of neural cells.

Histology is literally the study of tissues and is the origin of much of the scientific knowledge of internal structure and tissues (Hodgson & Bernard, 1992). There are many options for staining tissue, ranging from the cresyl violet stain which is a basic aniline dye, to immunohistochemical techniques which utilise antibodies such as TUNEL, used in the previous chapter.
The Nissl stain is appropriate in this situation, as neurons are well visualised and any alterations in morphology can be easily identified. The stain was named after Franz Nissl (1860-1919), a German neuropathologist and psychiatrist, as well as a close colleague of Alois Alzheimer.

Cresyl violet is used to stain Nissl substances, primarily composed of rough endoplasmic reticulum and nuclei. These substances are usually stained a dark blue or purple, depending on the protocol and neuronal morphology is thus easily visualised (Bauer, 1974).

5.2 Materials & Methods

5.2.1 Chemicals & Reagents

Quinolinic acid and DHEA were purchased from Sigma Chemical Company, Missouri. DPX was purchased from Philip Harris Ltd, England and Haupt's adhesive was prepared from 1g gelatine, 100ml water, 15ml glycerol and 2g phenol, added as a preservative. Cresyl violet was obtained from BDH Chemicals Ltd, England. Shandon Histoplast Wax was purchased from Lasec, South Africa. DHEA was prepared in 10% ethanol in ethyl oleate (vehicle) and QA was dissolved in PBS. All other chemicals and reagents were obtained locally and were of the highest quality available.

5.2.2 Animals

Animals were maintained as described in *Appendix A: Animal Care*. Animals were subjected to the same treatment regimen as described in Section 2.4.1.3. To summarise, the dosing regimen is provided in Table 5.1 below.

Table 5.1 Dosing regimen for histological investigation.

Group Name	Daily Subcutaneous Injection	Bilateral Intrahippocampal Injection on Day 8
Control	Vehicle	PBS
Toxin	Vehicle	120nmol Quinolinic acid
5mg/kg	DHEA in Vehicle	120nmol Quinolinic acid
10mg/kg	DHEA in Vehicle	120nmol Quinolinic acid
20mg/kg	DHEA in Vehicle	120nmol Quinolinic acid

5.2.3 Histological Techniques

5.2.3.1 Brain Fixation

Since neural issues are extremely fragile and easily susceptible to rapid anoxic and postmortem changes (Chang, 1995), whole rat brains were immediately immersed in Davidson's fixative for 48 hours as detailed in Section 4.2.3.1. The brains were then transferred to 70% ethanol for storage until further use.

5.2.3.2 Dehydration, Clearing and Waxing Process

Brains were embedded in wax for preservation and ease of sectioning as described in the previous chapter and laid out in Table 5.2 below.

Table 5.2 The table indicates the solutions utilised, as well as the appropriate duration of immersion, for embedding brains in paraffin wax for histological examination

Step		Processing Agent	Repeat X Time (Hrs)
1	Dehydration	50% Ethanol	1 x 2
2		70% Ethanol	1 x 2
3		80% Ethanol	1 x 2
4		90% Ethanol	1 x 2
5		96% Ethanol	2 x 2
6		Absolute Ethanol	3 x 2
7	Clearing	Absolute Ethanol: Chloroform	1 x 2
8		Chloroform	1 x 2
9		Xylene: Chloroform @ 60°C	1 x 1
10	Waxing	Melted Paraffin Wax (57-58°C mp) @ 60°C	1 x 1
11		Vacuum @ 60°C	15min
12		Melted Paraffin Wax (57-58°C mp) @ 60°C	2 x 1
13	Embedding	In molten wax @ 45°C	Overnight

5.2.3.3 Embedding

Brains embedded in wax were mounted onto wooden blocks and shaped as described in Section 4.2.3.2.

5.2.3.4 Sectioning

Sectioning was carried out as described in Section 4.2.3.5, with a slight modification. As mentioned above, the cresyl violet stain is a more robust technique than the TUNEL stain used in *Chapter 4: Apoptosis*, making the use of APES as a tissue adherent unnecessary. Haupt's adhesive is used instead. Sections of 5µm thickness were sliced into ribbons using a rotary microtome and placed into the water bath as described in Section 4.2.3.5.

The slides were painted with a fine layer of freshly prepared Haupt's adhesive before transferring sufficiently flattened ribbons of sections to slides. The slides were placed on a warming tray to allow water to evapourate, and then placed in an oven set at 45°C overnight to allow the tissue to adhere properly to the slide.

5.2.3.5 Deparaffinising Sections

As with the TUNEL stain used in *Chapter 4: Apoptosis*, the cresyl violet stain is hydrophilic. Consequently the tissue must first be dewaxed and rehydrated, reversing the entire embedding process required prior to sectioning. Due to the more robust nature of the stain as previously mentioned, an abbreviated dewaxing and rehydration protocol may be utilised. The paraffin was removed by running slides through xylene twice, followed by immersion in a mixture of equal parts of xylene and ethanol. Slides were then rinsed in ethanol and replaced in absolute ethanol overnight, as laid out in Table 5.3 below, before proceeding with cresyl violet staining.

Table 5.3 Dewaxing and rehydration protocol prior to Nissl staining.

Solvent	Duration
Xylene	5min X 2
Xylene:Ethanol	3min
Absolute Ethanol	5min
Absolute Ethanol	Overnight at 30°C

5.2.3.6 The Cresyl Violet Stain

The sections were Nissl stained using cresyl violet. This stains the Nissl substances intense purple and the nuclei purple, while leaving the background clear (Bauer, 1974).

Slides were removed from the absolute alcohol and placed in 0.1% cresyl violet solution for 2 hours. The solution was prepared from 0.25g cresyl violet, 0.0512g sodium acetate, 0.75ml glacial acetic acid and 250ml MilliQ water. The solution was corrected to pH 3.5 and filtered. Slides were removed from the cresyl violet stain and were rapidly differentiated in 95% ethanol by rinsing in a flat dish until the background was clear. The sections were then dehydrated as laid out in Table 5.4 below.

Table 5.4 Dehydration protocol for Nissl stained sections.

Solvent	Duration
Absolute Ethanol	5min X 2
Xylene	5min X 2

5.2.3.7 Microscopy

Prior to viewing slides, coverslips must be placed over the tissue to improve visibility and prevent damage. This was accomplished by first adding sufficient of the mountant, DPX, to each slide which had been kept moist with a small aliquot of xylene. Coverslips were then gently lowered into place. The slides were allowed to dry for 48 hours on a flat surface before viewing.

5.3 Results

All slides were viewed at 400 X magnification using a light microscope and images were captured with an Olympus digital camera.

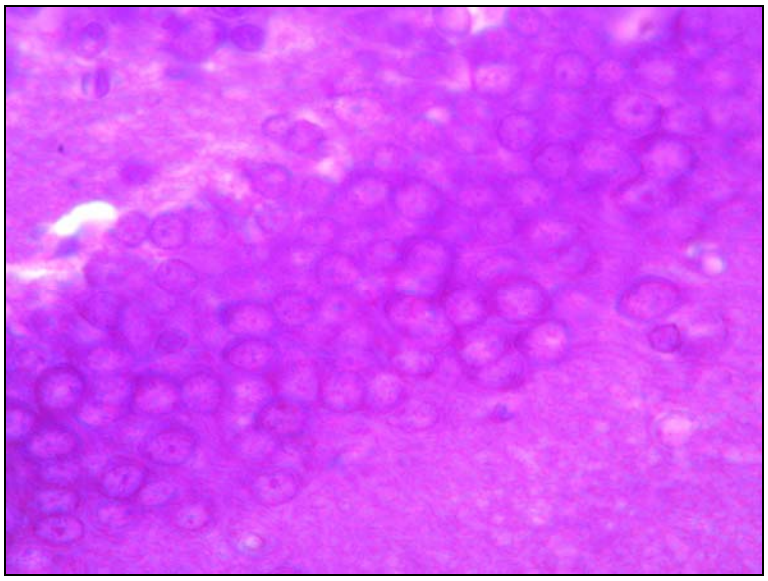


Fig 5.1 The photomicrograph of hippocampal tissue of rat receiving daily subcutaneous injections of the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained PBS alone (magnification X 400).

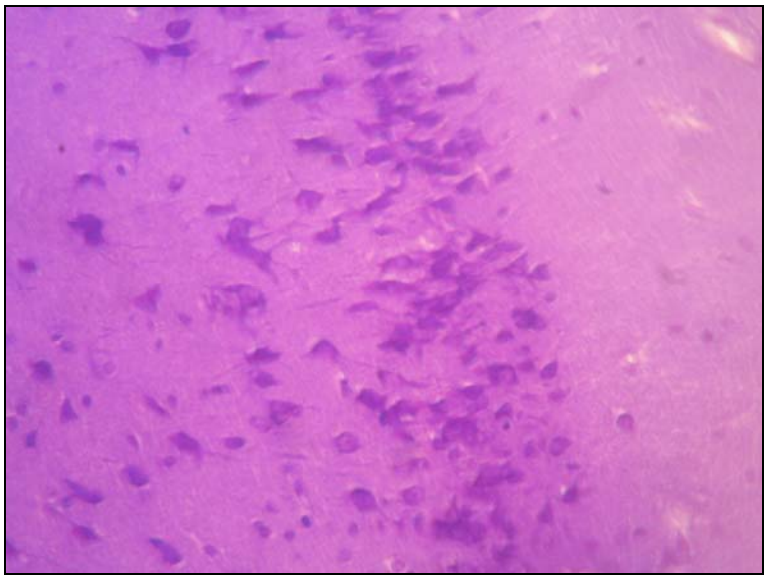


Fig 5.2 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 400).

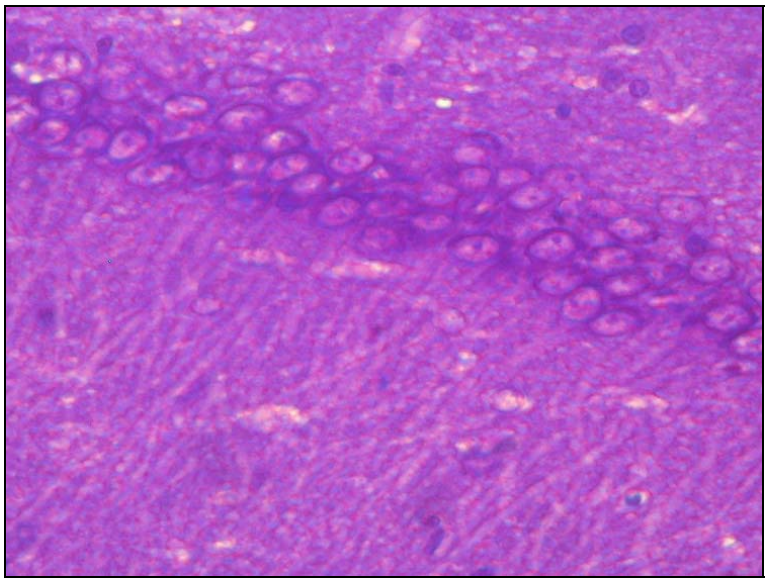


Fig 5.3 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 5mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 400).

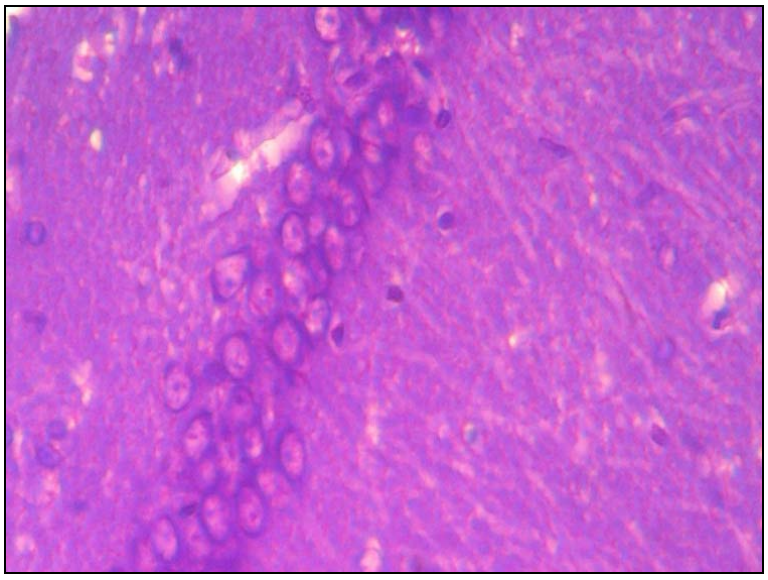


Fig 5.4 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 10mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 400).

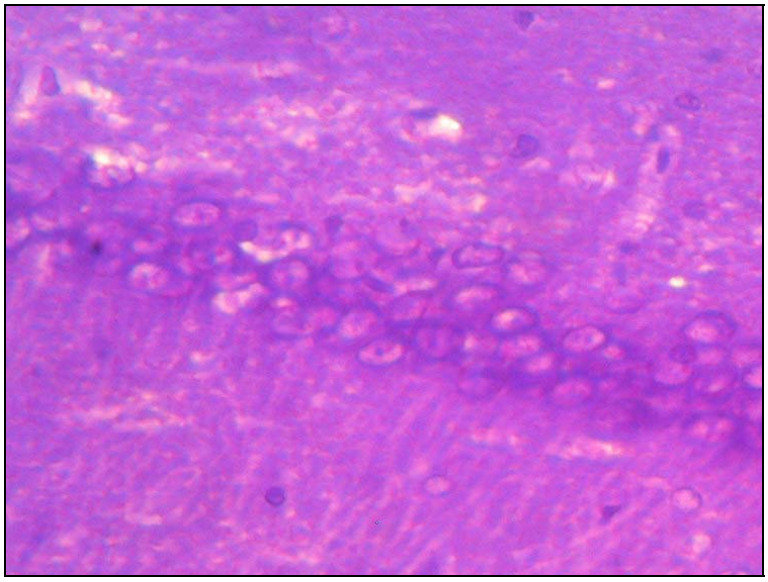


Fig 5.5 The photomicrograph is of the hippocampal tissue of rat receiving daily subcutaneous injections of 20mg/kg DHEA in the vehicle (10% ethanol in ethyl oleate). The intrahippocampal injection contained 120nmol QA (magnification X 400).

5.4 Discussion

The cells of Ammon's Horn have distinct pyramidal morphology which is altered with the introduction of a neurotoxin such as QA (Southgate, 1998). The cell bodies appear to be damaged which is characteristic of toxicity.

QA is an NMDA agonist as mentioned in Section 1.3.1 and causes damage by overstimulation of this receptor, resulting in increased cytosolic Ca^{2+} levels and eventual cell death (Santamaría *et al*, 2003). Thus, neurons damaged by QA typically exhibit swelling, alteration in the typical pyramidal morphology of hippocampal neurons and loss of cell integrity.

The results show that DHEA is protective of hippocampal neurons when administered according the above treatment regimen (see Table 5.1). Fig 5.2 shows hippocampal cells subjected to the neurotoxin QA with no subcutaneously administered DHEA. The cells do not display typical healthy morphology as exhibited in Fig 5.1 as QA causes significant damage and neurodegeneration, possibly due to a combination of osmotic changes and free radical damage, as discussed previously.

It is clear from the above photomicrographs (Figs 5.3 to 5.5) that DHEA has a beneficial effect on cell integrity, preserving the morphology of the cell in the presence of the NMDA agonist. These results are supported by *Chapter 2: Lipid Peroxidation Investigation* which demonstrated that DHEA reduced QA-induced lipid peroxidation, and thus cell membrane damage, in the hippocampus. These results are also in keeping with other investigations which demonstrated that other important and structurally similar hormones such as oestrogen and progesterone had similarly favourable effects on neural cells (Tomas-Camardiel *et al*, 2002; Garcia-Segura *et al*, 2003; Schumacher *et al*, 2003).

This concurs with the evidence provided in the previous chapter that DHEA had a beneficial effect on the induction of apoptosis by QA, reducing the apparent incidence of this type of programmed cell death.

DHEA could be protecting hippocampal neurons in several ways including inhibiting the production of free radicals, stabilisation of membranes or interacting with QA or one of its mediators.
Since DHEA(S) concentrations are minimal in rodents and can be considered naïve of endogenous DHEA (Baulieu, 1996), it is unlikely that this prevention of the harmful effects of DHEA is in fact due to the metabolic products of this hormone. However, further examination is required before this can be conclusively stated.

Pineal Organ Culture Studies

6.1 Introduction

Organ culture is a technique pioneered in 1926 in the Strangeways Laboratory in Cambridge (Strangeways & Fell, 1926). More than three quarters of a century later it is still a relatively simple yet effective technique for determining the effects of exogenous compounds on the endogenous synthesis of pineal indoleamines. Modifications to this technique have improved its scope immensely, from the initial observation of the growth of chick cartilage and eyes by Strangeways and Fell (Wilson, 2005) to the ability to keep fully differentiated organs alive while preventing their growth or further differentiation. The pineal gland in organ culture is able to remain viable for six days under optimal conditions, during which period it maintains it metabolic functions and can utilise exogenous serotonin to produce pineal indoleamines (Daya et al, 1989). As much as 95% of these synthesised indoles is secreted into the culture medium and may then be isolated and quantified.

Pineal indole metabolism has been well documented and the effect of DHEA on this metabolism may be a key to its properties as a neuroprotectant. The chief pineal hormone, melatonin, has been well-documented (Reiter et al, 1995) as a free radical scavenger and the effect of DHEA on its metabolism is of interest. Melatonin's antioxidant properties have resulted in the suggestion of a potential role in the prevention or alleviation of various neurological disorders.

Fundamentally, pineal glands are incubated with radiolabelled serotonin for 24 hours, in the presence or absence of DHEA. Thin Layer Chromatography (TLC) is utilised to separate the radiolabelled metabolites present in the culture medium and the amounts are determined using scintillometry.

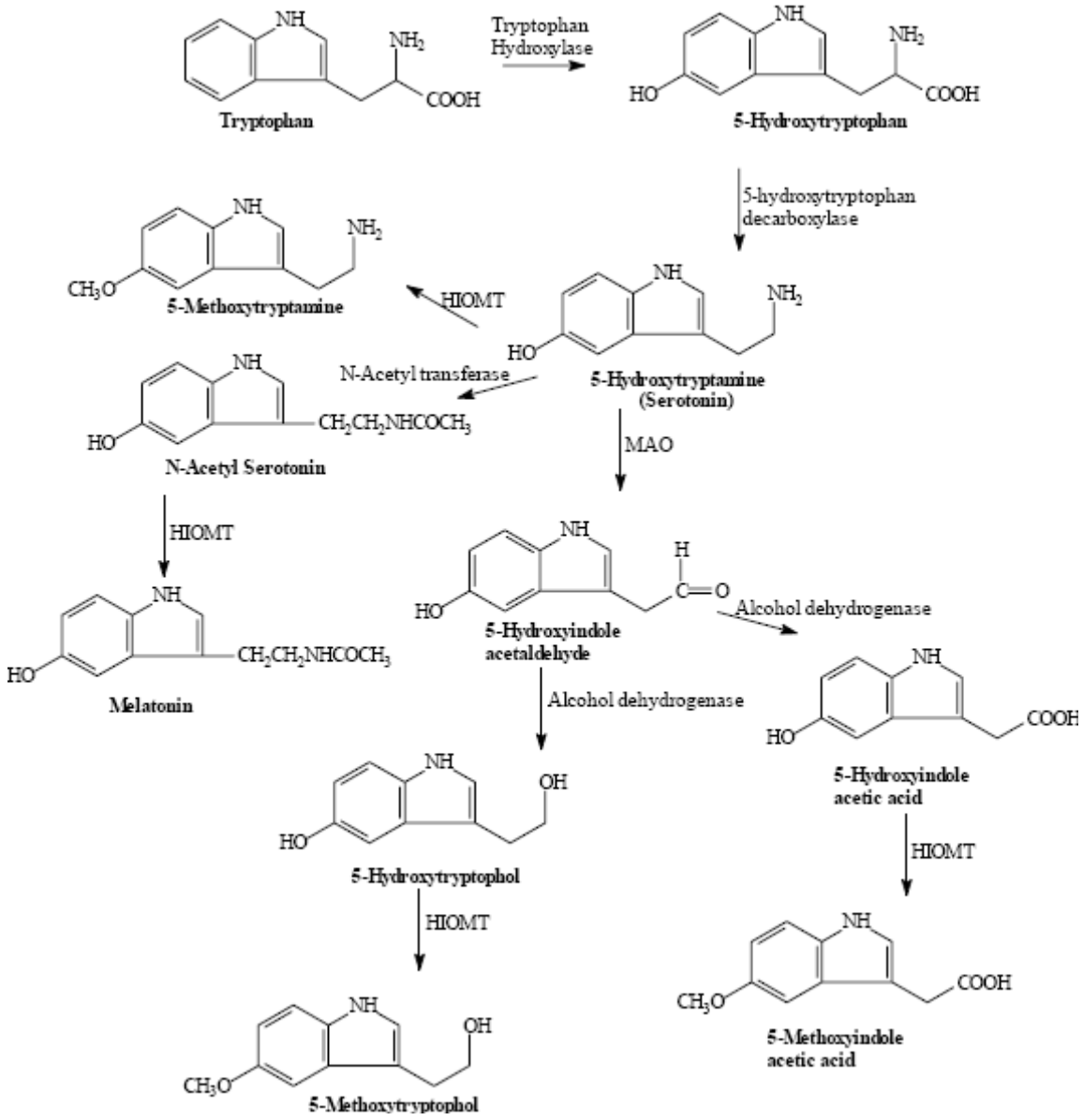


Fig 6.1 The diagram indicates the various metabolic pathways in pineal indole metabolism (modified from Young and Silman, 1982).

6.2 Materials & Methods

6.2.1 Chemicals & Reagents

DHEA was obtained from Sigma Chemical Co, St Louis, USA. ^{14}C Serotonin (specific activity: 55mCi/mL) was obtained from Amersham International, England. BGJb culture medium (composition shown below in Table 6.1) was obtained from Gibco, Europe and supplemented with the antibiotics streptomycin (0.1mg/L) and benzyl penicillin (100units/ml) (Hoechst, South Africa). The TLC plates (aluminium, coated with silica gel 60, Type F254 – 0.25mm) were purchased from Merck, Darmstadt, Germany. Beckman Ready-Sol Scintillation fluid was purchased from Beckman RIIC Ltd, Scotland. The indole standards HT, HA, HL, MA, ML, aMT, and aHT were purchased from Sigma Chemicals Co, St Louis, USA. All other reagents and chemicals were obtained locally and were of the highest purity available.

Table 6.1 Contents of the BGJb culture medium

Compound	Concentration (mg/litre)
Amino Acids	
L-Alanine	250.0
L-Arginine	175.0
L-Aspartic acid	150.0
L-Cysteine (HCL)	90.0
L-Glutamine	200.0
Glycine	800.0
L-Histidine	150.0
L-Isoleucine	30.0
L-Leucine	50.0
L-Lysine (HCL)	240.0
L-Methionine	50.0
L-Phenylalanine	50.0
L-Proline	400.0

L-Serine	200.0
L-Threonine	75.0
L-Tryptophan	40.0
DL-Valine	65.0
Vitamins	
α-Tocopherol phosphate	1.0
Ascorbic acid	50.0
Biotin	0.2
Calcium pantothenate	0.2
Choline chloride	50.0
Folic acid	0.2
Inositol	0.2
Para-aminobenzoic acid	2.0
Pyridoxal phosphate	0.2
Riboflavin	0.2
Thiamine hydrochloride	4.0
Vitamin B12	0.04
Inorganic Salts	
Dihydrogen sodium ortho-phosphate	90.0
Magnesium sulphate	200.0
Potassium Chloride	400.0
Potassium dihydrogen phosphate	160.0
Sodium bicarbonate	3500.0
Sodium chloride	5300.0
Supplements	
L – Glutamine	200
Streptomycin sulphate	0.1

Benzyl Penicillin	100 units/ml
Other Compounds	
Calcium lactate	555.0
Glucose	10000.0
Other Compounds cont.	
Phenol red	20.0
Sodium acetate	50.0

6.2.2 Animals

Male Wistar rats which had been cared for as laid out in *Appendix A: Animal Care*, weighing approximately 300g were randomly divided into two groups (n=5). These rats were killed by cervical dislocation at approximately 11h00.

6.2.3 Removal of Pineal Gland

After killing, the pineal glands were removed from the rat brains by making incisions on both sides of the skull, reaching from the foramen magnum to the orbits. The top of the skull was carefully lifted and the pineal gland exposed. The pineal gland was isolated and all adhering tissue removed.

6.2.4 Organ Culture

The technique employed by Klein and Notides (1969) was modified to achieve separation of the radiolabelled indoleamines. The pineal glands were immediately placed in sterile 75 x 10mm Kimble tubes containing 52µl of fortified BGJb culture medium. In addition, these tubes contained either 5µl of the test solution of DHEA to produce the final desired concentration of 1×10^{-5} M, or 5µl of the vehicle of the test solution as a control group as shown below.
Subsequently, 8µl of ^{14}C Serotonin with a specific activity of 55mCi/mL was added to each tube, producing a total volume of 65µl.

Table 6.2 The composition of the liquid medium used in the Kimble tubes in control and DHEA-treated samples.

Control	Volume	Test
BGJb Culture Medium	52µl	BGJb Culture Medium
Vehicle (absolute ethanol)	5µl	DHEA test solution
^{14}C Serotonin	8µl	^{14}C Serotonin
	65µl	

The Kimble tubes were then saturated with carbogen (5% carbon dioxide in oxygen) and sealed, before incubating at 37°C in the dark for 24 hours in a Forma Scientific Model 3028 incubator. The reaction was halted by the removal of the pineal gland from the solution. The medium was then frozen and stored at -20°C until TLC analysis was performed.

6.2.4.1 Spotting of the TLC Plates

TLC plates first activated by heating them at 70°C for 1hour were spotted with culture medium and dried under a thin stream of nitrogen gas to prevent atmospheric oxidation of the metabolites and hasten drying. The standard solution was prepared by adding 1mg of each of the seven indoleamines (HT, HA, HL, MA, ML, aMT, and aHT) to a solution of 95% ethanol, containing 1% ascorbic acid to prevent undesirable oxidation. This standard solution was then delicately spotted over the culture medium and again dried under a thin stream of nitrogen. The plates were spotted under subdued lights to prevent photooxidation of the indoleamines.

6.2.4.2 Elution of the TLC Plates

Two solvent systems were utilised, namely Solvent System A, consisting of chloroform:methanol:glacial acetic acid in a ratio of 93:7:1, and Solvent System B comprising ethyl acetate.

The plate was eluted in the Solvent System A twice. During each elution, the solvent front was allowed to develop a distance of 9cm, after which the plate was removed from the TLC tank and dried under a gentle stream of nitrogen, before the subsequent/second elution commenced. The

plate was then turned at right angles (90°) to the first direction of development and eluted in Solvent System B. Once again the plate was dried under nitrogen gas before visualizing the spots under UV light at 254nm.

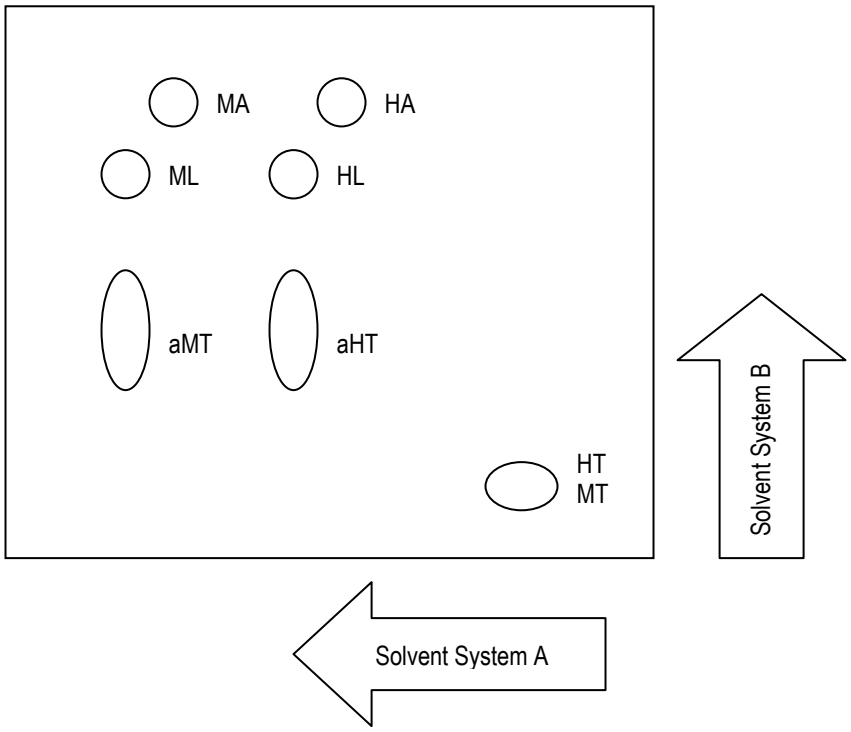


Fig 6.2 Diagrammatic representation of a TLC plate when visualised under UV light, showing separation of the indoleamines. Each plate is eluted twice in the direction indicated as "Solvent System A", then once more in the direction indicated as "Solvent System B" in order to achieve satisfactory separation of the standards MA, HA, ML, HL aMT and aHT.

6.2.4.3 Determining the Radioactivity of Metabolites

The spots were isolated from the plates and added to individual plastic scintillation vials. Ethanol (1ml) and the scintillation cocktail (3ml) were added to each vial and after sealing, the vials were vortexed for thirty seconds to ensure the indoleamines were homogenously mixed with the solvent system, and did not remain on the TLC plate fragments.

The radioactivity of each metabolite was determined using a Packard Tri-Carb scintillation counter, model number 2300TR.

6.2.4.4 Statistical Analysis

All data was first analysed using ANOVA, followed by the Student-Newman-Keuls Multiple Range Test. Comparisons between mean values were significantly different if *p* values were <0.05 (Zar, 1974).

6.3 Results

Results were expressed as counts per minute per 10 µL per pineal gland, with n=5.
Serotonin and methoxytryptamine do not migrate from the origin in this solvent system (Klein & Notides, 1969), thus results expressed are at the origin are for both these compounds.
Good separation of the indole metabolites was achieved and high counts were obtained.
Background counts were negligible.

Table 6.3 The effect of *in vitro* DHEA supplementation on rat pineal indole metabolism. * indicates a significant difference from the control with $p<0.001$ (Student-Newman-Keuls Multiple Range Test).

	Mean ± SEM (n=5)	
Pineal Metabolite	Control	DHEA
Melatonin (aMT)	141 ± 53	115 ± 57
5-methoxytryptophol (ML)	136 ± 45	91 ± 40
5-methoxyindoleacetic acid (MA)	52 ± 11	168 ± 34
N-acetyl serotonin (aHT)	877 ± 269	3229 ± 867 *
5-hydroxytryptophol (HL)	2284 ± 621	7229 ± 1593
5-hydroxyindoleacetic acid (HA)	3269 ± 1046	11214 ± 2867
Origin (HT/MT)	34221 ± 4450	41351 ± 9205 *

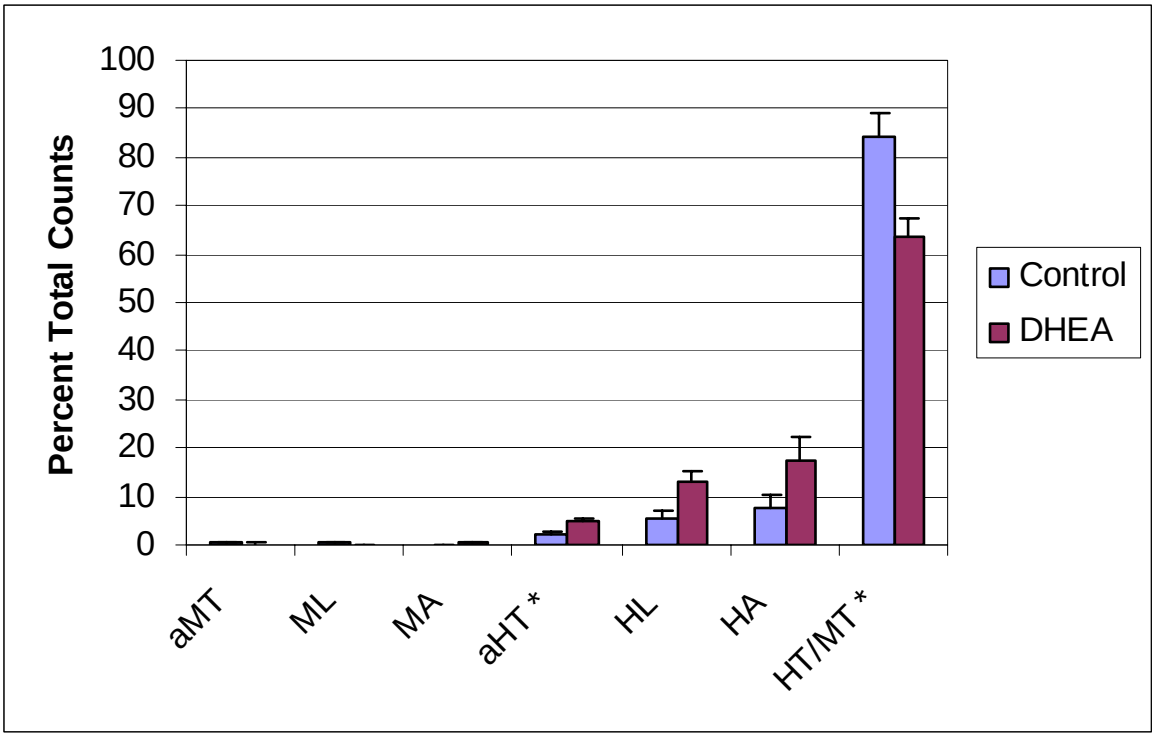


Fig 6.3 The effect of *in vitro* DHEA supplementation on rat pineal indole metabolism. Results are expressed as percentage total count per minute per 10 µL per pineal gland.

* indicates a significant difference from the control with $p<0.001$ (Student-Newman-Keuls Multiple Range Test). The x axis indicates the metabolite.

The results show a significant ($p<0.001$) decrease in the amount of HT/MT in pineal glands incubated with DHEA compared with the control and a significant ($p<0.001$) increase in the production of N-acetyl serotonin. There was no significant increase in the production of MA HL HA as well as no significant decrease in the production of the remaining metabolites, ML and melatonin.

6.4 Discussion

The rat pineal gland, when subjected to organ culture, metabolises radiolabelled serotonin to various pineal indoleamines. The effect of an exogenous substance on this metabolism can then be assessed by comparing drug treated pineals to untreated ones.

From the above graph, it can be observed that while DHEA does have an impact on certain metabolites, there is no significant change in the synthesis of melatonin, a well-documented free radical scavenger and anti-oxidant. These results concur with those of Djeridane and Touitou (2004), confirming that the neuroprotectant effect of DHEA, as outlined in *Chapter 2: Lipid Peroxidation Investigation*, is not due to an increase in the production of melatonin. San Martin and Touitou (2000) previously demonstrated that DHEAS increased -adrenergic-stimulated melatonin secretion by rat pineals *in vitro.*

The results show that the presence of DHEA results in a significant decrease in the amount of serotonin/methoxytryptamine, suggesting that the overall pineal indole metabolism is increased, leaving less substrate after 24 hours of organ culture. This is plausible, since an increase in metabolism would not affect the relative amounts of metabolites produced.

DHEA significantly increases the production of N-acetylserotonin, although a subsequent increase in the amount of melatonin synthesised is not evident. This indicates that while more serotonin is converted to N-acetylserotonin via N-acetyltransferase (NAT), DHEA does not cause an increase in the activity of hydroxyindole-O-methyltransferase (HIOMT). This is supported by the fact that other metabolites relying on HIOMT for synthesis, namely 5-methoxytryptamine, 5-methoxyindole acetic acid and 5-methoxytryptophol, show no significant increase in production (see Fig 6.4). Djeridane & Touitou (2004) showed that neither DHEA nor its sulphated derivative alter HIOMT over a range of acutely administered doses, while DHEAS results in increased melatonin production through increased activity of NAT. This enzyme is in most circumstances the rate-limiting step in melatonin synthesis (Klein *et al*, 1997) and is subject to a circadian rhythm (Klein & Weller, 1970).

Methods including drying spots under nitrogen gas, the addition of ascorbic acid to the standard solution, and subdued lighting were employed to minimise undesirable oxidation of the metabolites which could have affected results.

These results are derived from an *in vitro* study and it needs to be determined whether the *in vivo* administration of DHEA would produce similar effects. The question concerning the existence of an *in vitro-in vivo* correlation bears further investigation.

Metal Interaction Studies

7.1 Introduction

Previously it has been established that DHEA is able to reduce QA-induced lipid peroxidation, as well as apoptosis and other structural damage. Since the mechanism of action of DHEA is unclear, and metal ions have a well-established role in neurodegenerative processes (Bear et al, 2003) this chapter will explore the possibility that DHEA interacts with metal ions.

Various techniques may be utilised in the characterisation of metal-ligand interactions including ultra-violet/visible (UV/VIS) spectroscopy, infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) and electrochemistry.

Since the mechanism of action of DHEA with regard to neuroprotection is largely unknown, and metal ions are known to be important in the generation of free radicals, the investigation of a possible interaction between DHEA and two biologically important ions, namely Cu(II) and Fe(III) was conducted. Two methods were employed. Firstly, a UV/VIS spectroscopic study was performed to determine if an interaction was occurring, then these results were confirmed and expanded upon through an electrochemical investigation.

7.2 Characterisation of Metal-Ligand Interaction Using UV/VIS Spectroscopy

7.2.1 Materials & Methods

7.2.1.1 Chemicals & Reagents

DHEA was obtained from Sigma Chemicals Co, St Louis, USA. Iron chloride and copper chloride were purchased from Merck, Midrand, South Africa. DHEA was dissolved in 30% ethanol due to its poor aqueous solubility. All reagents were prepared in de-aerated MilliQ water unless otherwise noted. All other chemicals and reagents were obtained locally and were of the highest quality available.

7.2.1.2 UV/VIS Spectroscopy

Spectroscopic analyses are based on the theory that compounds posses the ability to absorb electromagnetic radiation at specific wavelengths. Since all compounds containing alternating double and single bonds will absorb UV radiation, and all coloured compounds absorb visible radiation, UV/VIS spectroscopy can be widely utilised. These absorption spectra are characteristic and any alteration in the structure or composition of a compound will result in a change in the spectrum of that compound, usually by a shift in wavelength or a change in extinction coefficient of the absorbance (Newman, 1969). By analysing the absorption spectrum of DHEA alone in solution, as well as the effect of adding increasing concentrations of metal ion, the interaction between DHEA and the two metals under investigation can be characterised. A range of concentrations of DHEA and metal were investigated from 1×10^{-3} M to 1×10^{-6} M. Samples were analysed using a GBC UV/VIS 916 spectrophotometric detector.

7.2.2 Results

The results are summarised below in Table 7.1. The lambda max for DHEA is 292.4nm. At the DHEA-Fe(III) ratio of 1:1, the peak height shifts to a shorter wavelength of 288.40nm. At the DHEA-Fe(III) ratio of 10:1, the peak wavelength is 289.2nm and at concentrations above 10:1, the peak wavelength shifts to 290nm and remains constant. The absorbance demonstrates a slight increase, but this change is not sufficient to be significant. This trend is repeated for DHEA-Cu(II)

experiment where above 10:1 the peak wavelength shifts to 292.4nm and remains constant for higher ratios.

Table 7.1 The table shows the relative concentrations of DHEA and metal ions, as well as the effect on peak height in terms of wavelength and absorbance.

Concentration of DHEA (mM)	Concentration of metal (mM)	Peak height (Absorbance)	Peak Wavelength (nm)
	Fe(III)		
1	1	0.961	288.4
10	1	0.387	289.2
100	1	0.670	290.0
	Cu(II)		
1	1	0.853	291.1
10	1	0.701	290.0
100	1	0.614	290.0

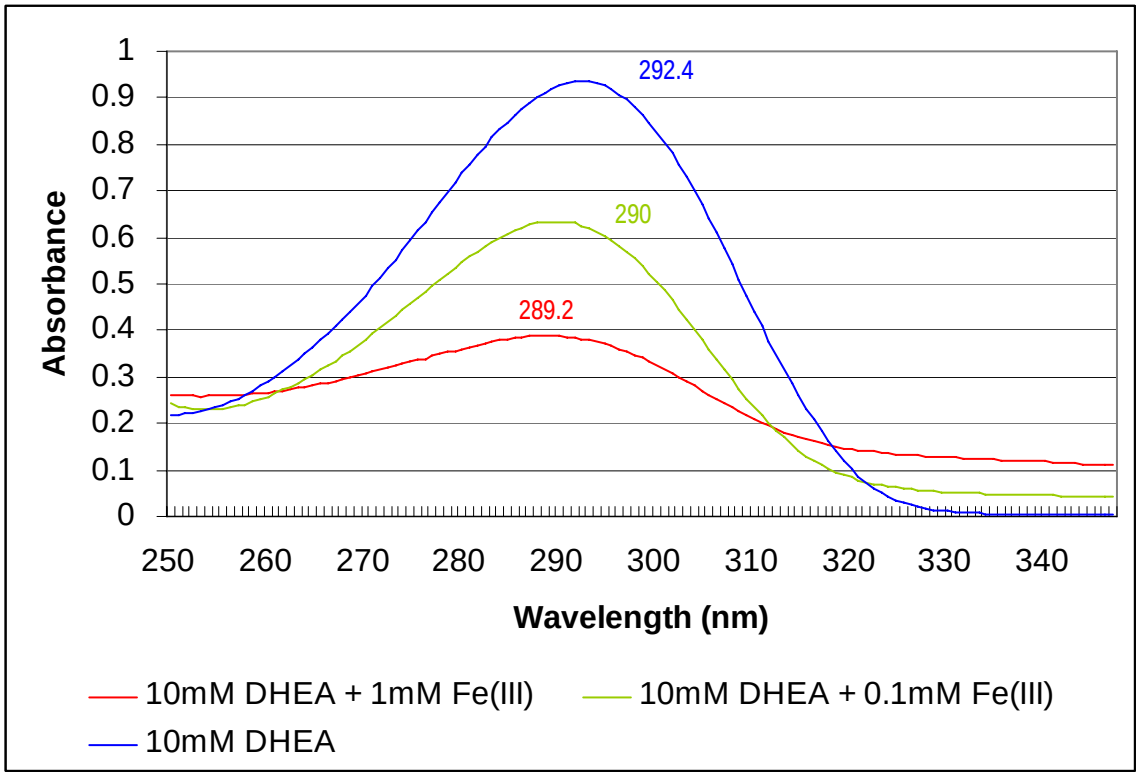


Fig 7.1 The graph shows the reduction in peak height and the shift to shorter wavelengths of DHEA upon the addition of Fe(III).

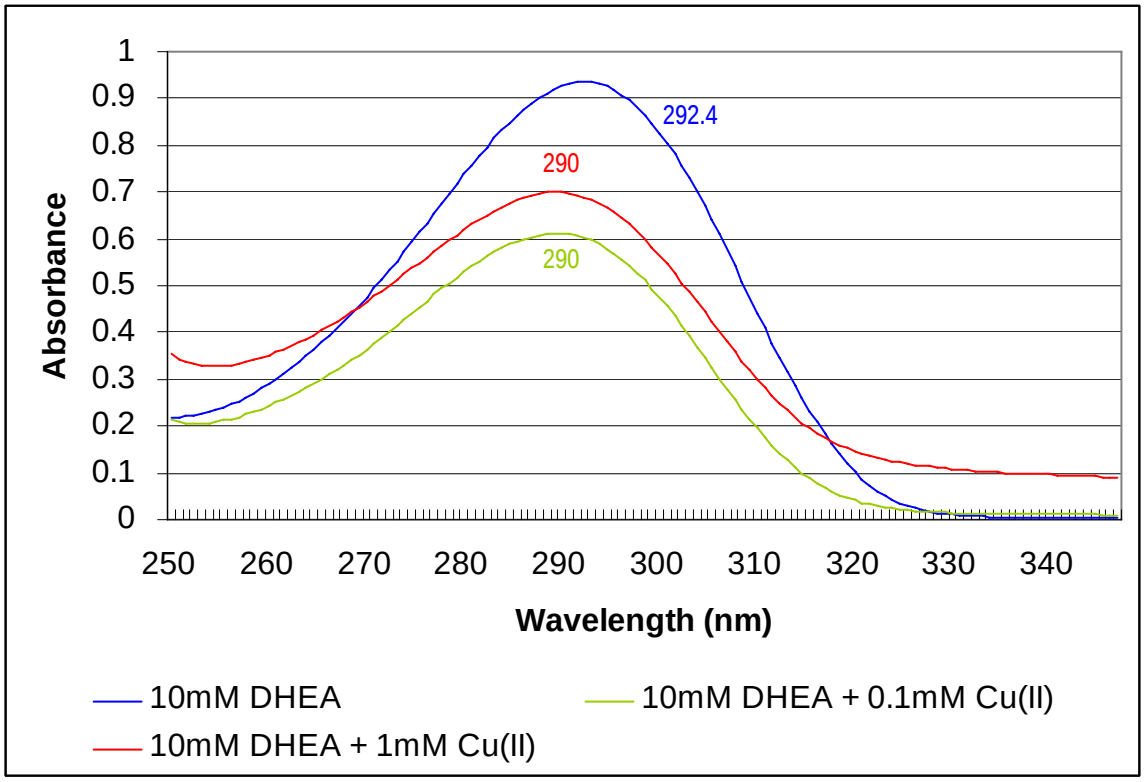


Fig 7.2 The graph shows the reduction in peak height and the shift to shorter wavelengths of DHEA upon the addition of Cu(III), although at ratios above 10:1, there is no further shift in wavelength.

7.2.3 Discussion

When DHEA and Fe(III) are present in equimolar amounts, there is a distinct blue shift, although the reduction in absorbance is not marked. When the concentration of DHEA exceeds the concentration of Fe(III) by a factor of ten, this shift to a shorter wavelength of radiation is still evident. The reduction in absorbance and the peak shift to shorter wavelengths displayed by DHEA in the presence of Fe(III), as well as the presence of Cu(II) may be indicative of a weak metal-ligand interaction. Electrochemical investigations as detailed below will further examine the hypothesis that DHEA may have a weak interaction with Cu(II) and Fe(III).

7.3 Characterisation of Metal-Ligand Interaction Using Electrochemistry

7.3.1 Materials & Methods

7.3.1.1 Chemicals & Reagents

DHEA was obtained from Sigma Chemicals Co, St Louis, USA. Iron chloride and copper chloride were purchased from Merck, Midrand, South Africa. DHEA was dissolved in 30% ethanol in MilliQ water. Tris-HCl was used to prepare a buffer of pH 7.4.with 30% ethanol. All reagents were prepared in de-aerated Milli-Q water unless otherwise noted. All other chemicals and reagents were obtained locally and were of the highest quality available.

7.3.1.2 Electrochemistry

Electrochemistry has been used successfully in several metal-ligand investigations including those using endogenous molecules as ligands (Limson *et al*, 1998; Limson & Nyokong, 1997; Lack & Nyokong, 2001). The investigation of electrochemical interactions was conducted in this study using an electrochemical cell comprising three electrodes, illustrated in Fig 7.3 below.

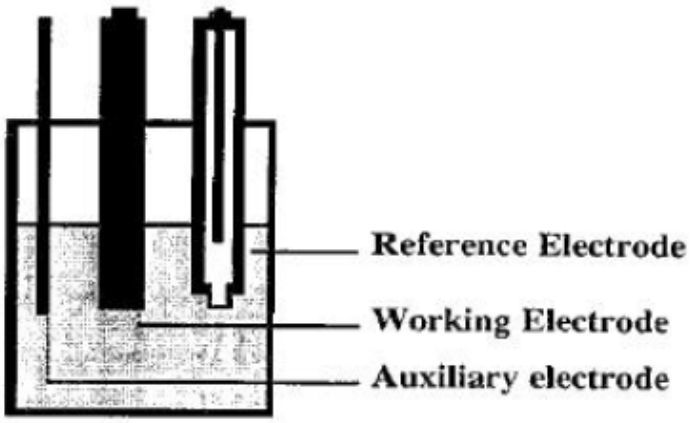


Fig 7.3 A diagrammatic representation of the cell used in electrochemical analyses, showing the reference, working and auxiliary electrodes (Limson, 1998).

The primary electrode is the reference electrode against which the potential of the working electrode is measured. The reference electrode maintains a constant potential and is isolated. Various reference electrodes exist including the hydrogen electrode, smooth platinum electrode,

saturated calomel electrode, glass electrode and the silver/silver chloride electrode. The silver/silver chloride (Ag/AgCl) electrode is particularly useful in aqueous solutions and comprises a silver wire anodised with silver chloride. The wire is encased in a glass tube containing concentrated solutions of AgCl and a chloride salt, usually potassium or sodium. The electrode is protected from solution by the semi-permeable salt bridge (Sole, 1995; Hawkridge, 1996).
The auxiliary electrode is constructed of an inert metal such as platinum, mercury or gold (Glasstone, 1942). It prevents voltage drop across the working and reference electrodes. Glassy carbon electodes (GCE) are frequently utilised as working electrodes since they posess suitable mechanical and electrical properties. They are chemically inert, have a low electrical resistance and performance is reproducible. It is at the working electrode that the analyte undergoes oxidation and reduction.

Two types of scans are frequently employed to asses the electrochemical activity of compounds. These are adsorptive stripping and cyclic voltammetry. Both are obtained from the same apparatus as described above. Adsorptive stripping voltammetry (ASV) is a useful technique for assessing metal-ligand interactions since analytes tend to naturally pre-concentrate at an electrode and by polarising the electrode at a more negative potential than the reduction potential of the metal, this tendency is enhanced.

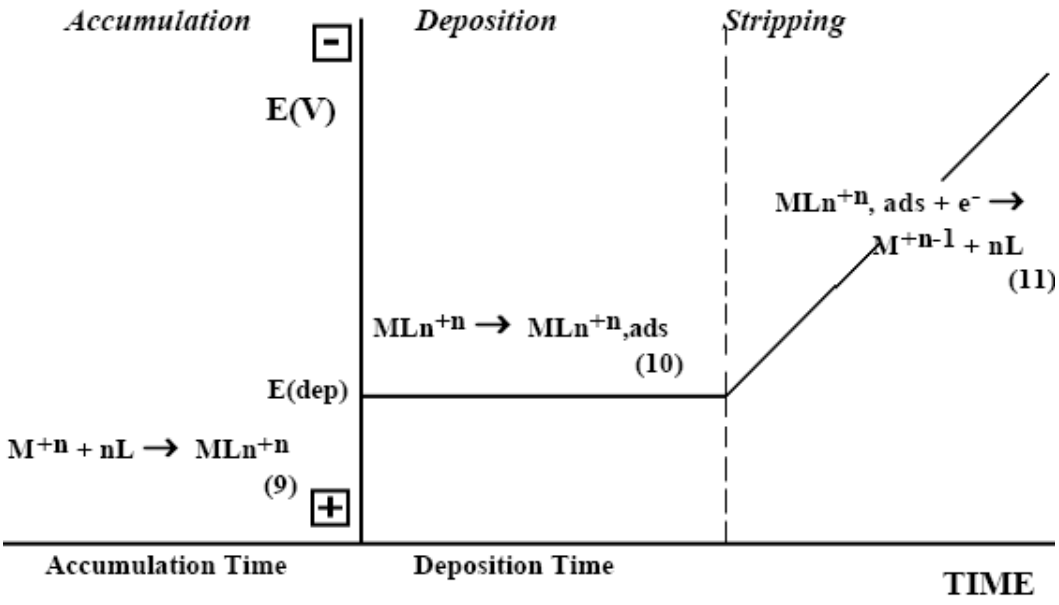


Fig 7.4 The diagram illustrates the adsorptive stripping voltammetric technique, including accumulation, deposition and stripping. The equations show the reactions occurring at the electrode. When a suitable chelating ligand (L) is added to a solution of electrolyte and metal analyte (M), metal-ligand (ML) complexation is facilitated and this complex is adsorbed at the electrode during the deposition time (Limson, 1998).

A metal ion will result in a specific response at a given potential, providing a specific voltammogram. The addition of a ligand facilitates the metal ion movement to the electrode, thereby changing the profile of the voltammogram. An increase in current indicates affinity of the ligand toward the metal, while a decrease in current response may indicate competition between the ligand and analyte, or the formation of a strong metal-ligand bond which is not easily reduced. A negative potential shift is characteristic of a strong metal-ligand interaction, while a large positive shift is associated with a weak metal-ligand interaction (Limson *et al*, 1998; Lack & Nyokong, 2001).

Tris-HCl at pH 7.4 was used in the preparation of the cells which were made up with metal-ligand ratios from 1 to 6 (hereafter referred to as ML1 to ML6) and analysed electrochemically. DHEA was prepared in 30% ethanol due to its poor aqueous solubility. Nitrogen gas is bubbled through the cell prior to scanning to remove traces of oxygen, thus increasing the rate of evapouration of

the ethanol solution. Evapouration efficiency may affect the reduction-oxidation of metal ions since these processes are a function of temperature. Removal of oxygen using an inert gas is essential as oxygen is electroactive at most electrodes and may interfere with voltammetric and electrochemical measurements (Sawyer & Roberts, 1974). Fresh cells were prepared prior to each scan in order to prevent loss of solvent which may have had an effect on the results.

The Bio Analytical Systems CV-50W voltametric analyser and C2 cell stand were used to maintain a constant atmosphere (BAS, Indiana, USA). The working, auxiliary and reference electrodes employed for voltammetric investigations were the glassy carbon electrode (3mm), platinum wire and a silver/silver chloride [KCl = 3M] electrode, respectively. The GCE was polished prior to each use with alumina on a Buehler pad, followed by rinsing with nitric acid and Milli-Q water. The purging time, deposition potential and deposition time were kept constant for the adsorptive stripping voltammograms of each metal. Voltammograms were scanned in the negative direction from the deposition potential to at least 0.5V beyond the reduction of the metal at a scan rate of $0.10V.s^{-1}$ to strip the adsorbed species from the electrode. During stripping, current responses to the reduction of the metal-ligand species were measured as a function of potential. All potential values quoted are referenced against the Ag/AgCl reference electrode.

7.3.2 Results

7.3.2.1 Copper(II)-DHEA Investigation

The figures show the decrease in current at the electrode (Fig 7.5) when the DHEA concentration is equal to the Cu(II) concentration (ML1) and when it is quadruple the metal concentration (ML4), as well as the shift to a slightly more negative potential (Fig 7.6). Fig 7.7 combines these data to demonstrate that increasing concentrations of DHEA are exerting an effect on the profile of the voltammogram.

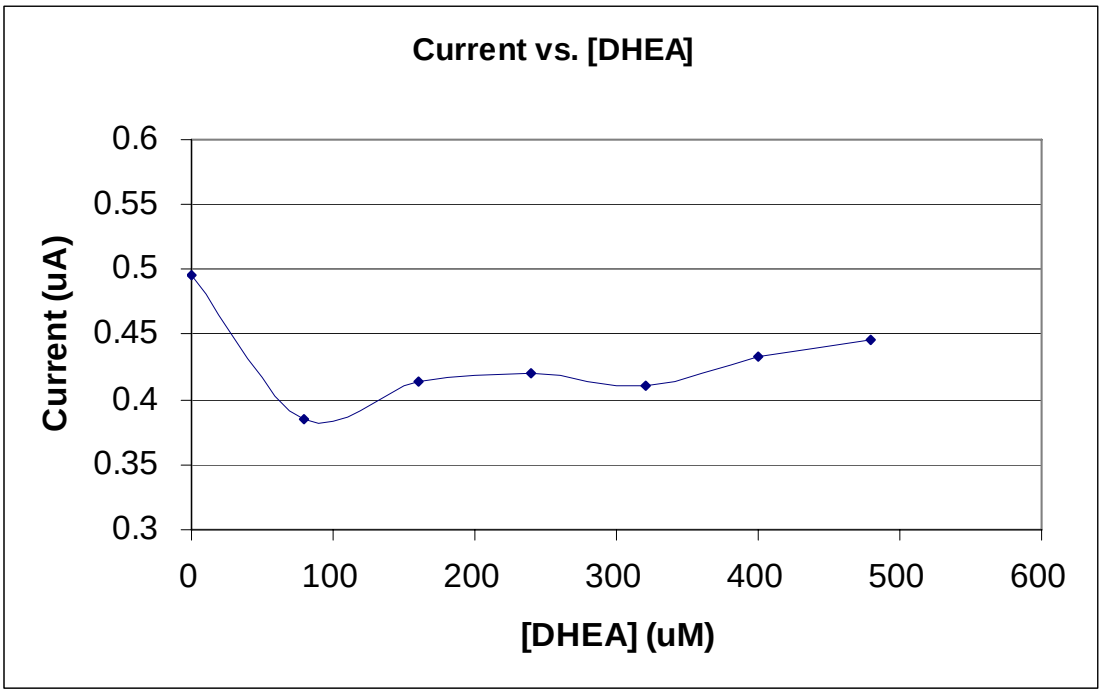


Fig 7.5 The graph shows the effect of increasing concentration of DHEA on the change in current in the electrochemical cell. The troughs in the graph at ML1 and ML4 are indicative of an interaction with Cu(II).

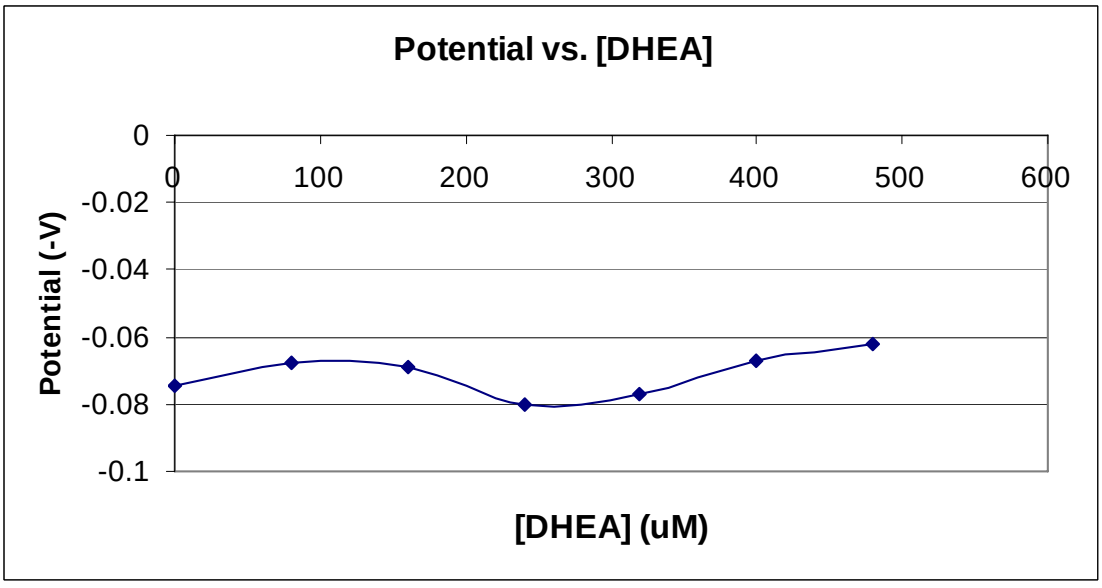


Fig 7.6 The graph shows the effect of increasing concentration of DHEA on the shift in potential in the electrochemical cell. The changes in the graph correspond with those in Fig 7.5 above.

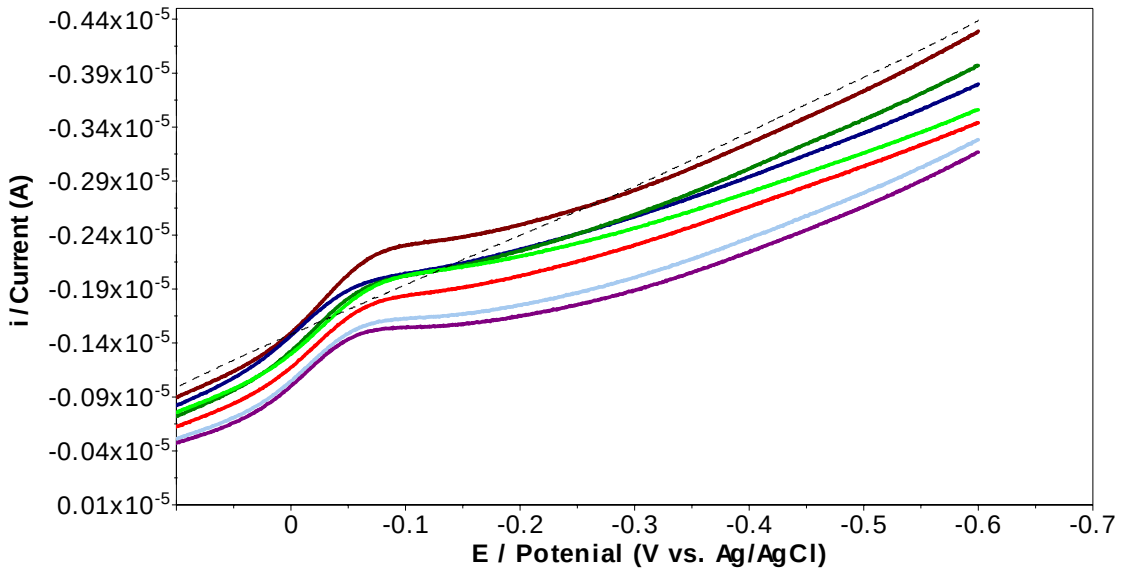


Fig 7.7 The voltammogram illustrates the interaction between DHEA and Cu(II) in Tris-HCl, pH 7.4. Each line represents All cells contained 80µM Cu(II). The DHEA concentration in each cell is 0µM (dark red), 200µM (dark blue), 400µM (dark green), 600µM (light green), 800µM (light red), 1000µM (magenta), 1200µM (light blue). The copper concentration is 200µM throughout. The baseline is represented by the dashed black line. The vertical axis represents the current, while the horizontal axis shows the potential versus the Ag/AgCl electrode.

7.3.2.2 Iron(III)-DHEA Investigation

The figures below indicate at decrease in current (Fig 7.8) and a shift to a more negative potential on the addition of DHEA (Fig 7.9) These data are combined in Fig 7.10 to illustrate the effect of increasing concentrations of DHEA on the profile of the voltammogram.

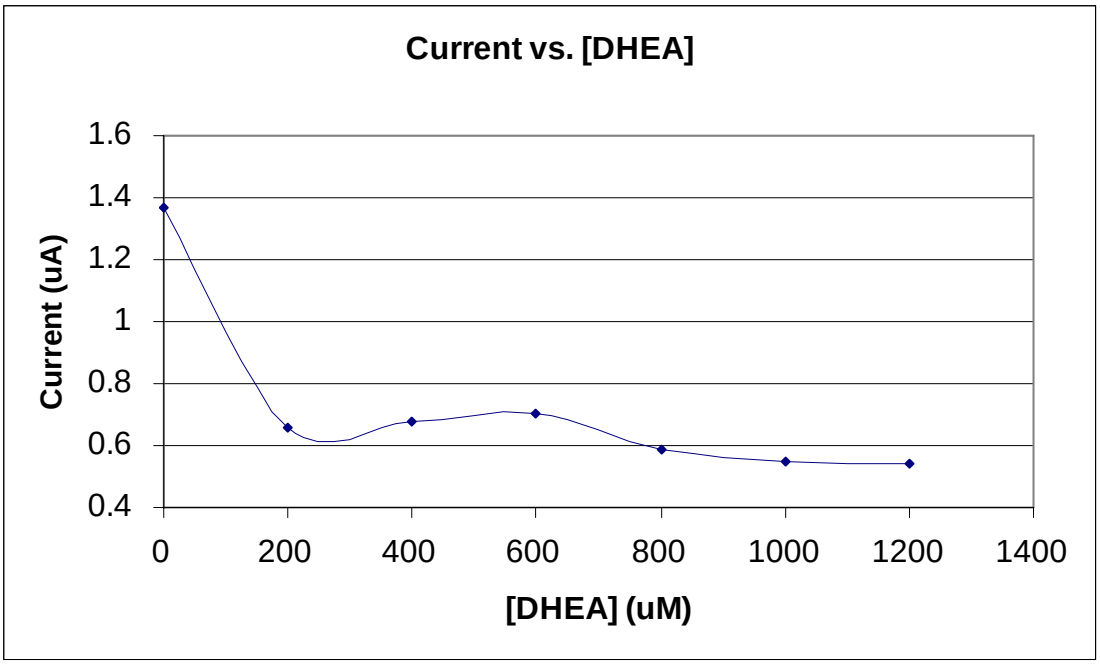


Fig 7.8 The graph shows the effect of increasing concentration of DHEA on the change in current in the electrochemical cell containing Fe(III).

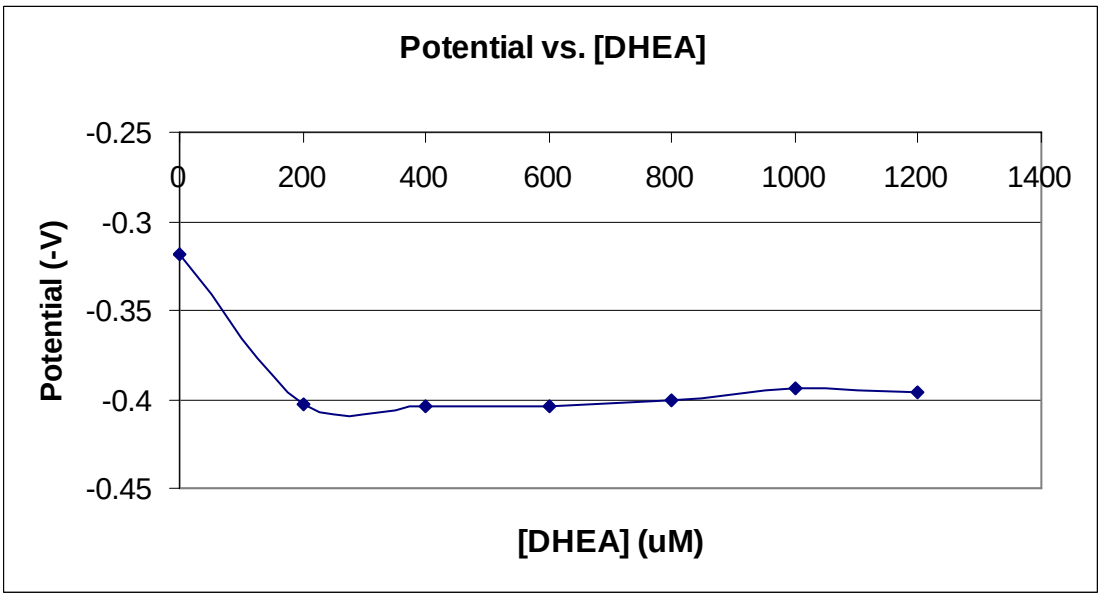


Fig 7.9 The graph shows the effect of increasing concentration of DHEA on the shift in potential in the electrochemical cell. The changes in the graph correspond with those in Fig 7.8 above.

In Fig 7.10 below the shift in potential is apparent from -0.32V for Fe(III) alone to -0.4V in the presence of equimolar DHEA. There is little variation in potential for the increased concentrations of DHEA examined.

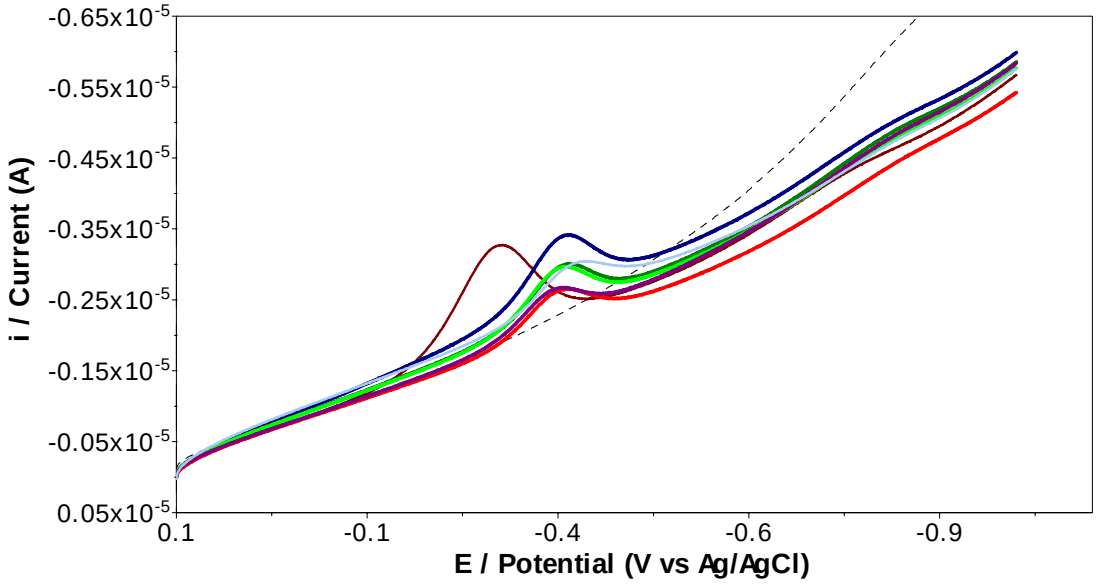


Fig 7.10 The voltammogram illustrates the interaction between DHEA and Fe(III) in Tris-HCl, pH 7.4. The DHEA concentration in each cell is 0µM (dark red), 80µM (dark blue), 160µM (dark green), 240µM (light green), 320µM (light red), 400µM (magenta), 480µM (light blue). The Fe(III) concentration is 80µM throughout. The baseline is represented by the dashed black line. The vertical axis represents the current, while the horizontal axis shows the potential versus the Ag/AgCl electrode.

7.3.3 Discussion

The results show that DHEA, while not electrochemically active itself, is interacting with both metal ions examined. A scan of DHEA alone shows the steroid exhibits no electrochemical activity up to concentration of 1 x 10^{-3}M. There is evidence of visible precipitation from 3 x 10^{-3} M.

The addition of DHEA to Fe(III) results in a shift to more negative potential with a minimal change in peak height. The peak observed for DHEA and Fe(III) at -0.4V suggests a metal ligand interaction since this potential is more negative than metal alone (Fig 7.10). The reduction in peak and shift to more negative potential indicates Fe(III) is becoming more difficult to reduce, which is a function of ligand interaction. Subsequent scans of increasing concentrations of DHEA show a successive shift to a more positive current. This is an indication that the extent of interaction is not

marked, but that a weak interaction does exist. Cu(II) exhibits a negligible shift in potential, but the successive shift to more positive current is still apparent. This suggests that the affinity and strength of the interaction are both weak.

It has been noted that the acidity of the solution, as well as the concentration of species under consideration will have an effect on potential (Butler, 1964). Since any neuroprotective effects exerted by DHEA will occur at physiological pH, it is irrelevant to this investigation how DHEA may interact with metal ions at non-physiological pH. In addition, chloride ions form fairly stable complexes with many metal ions so Burgess (1999) recommends avoiding hydrochloric acid in such investigations, but again, chloride ions are present physiologically.

DHEA was able to reduce QA-induced lipid peroxidation in both *in vitro* and *in vivo* investigations. It has been suggested that the peroxidative effect of QA is mediated by metal ions, particularly iron (Stipêk *et al*, 1996). Elevated levels of iron have been demonstrated in various neurodegenerative disorders, including AD, Parkinson's disease, multiple system atrophy and progressive supranuclear palsy (Campbell, 2001). The interaction of DHEA with Fe(III), albeit weak, may provide a clue to the unclear mechanism of action of DHEA, even though QA interacts with ferrous and not ferric ions. Further investigation of the interaction of this steroid molecule with metal ions will be of value.

Summary

Chapter 1

This literature review provides a concise introduction to dehydroepiandrosterone and relevant theory relating to neuroscience. Areas such as basic neuroanatomy were covered. Neurotoxins such as cyanide and quinolinic acid were introduced and the theory of excitotoxicity was explored. The role of free radicals in the biological system was addressed and the chapter also covered how cell death occurs. A brief discussion on neurodegeneration and neuroprotection was followed by a comprehensive review of dehydroepiandrosterone including known pharmacokinetics and pharmacology. The concepts of neurosteroids and neuroactive steroids were introduced, before dealing with the mechanism of action of dehydroepiandrosterone.

Chapter 2

Quinolinic acid is well established as a neurotoxic substance and Chapter 2 illustrated the effect of quinolinic acid on rat brain homogenate. Increasing concentrations of QA were shown to cause an increase in lipid peroxidation in a dose-dependent manner. These results concur with previously reported findings. The effect of DHEA on QA-induced lipid peroxidation was investigated in both *in vitro* and *in vivo* models and the results show that DHEA reduces QA-induced lipid peroxidation. Data obtained *in vivo* does not suggest a dose-dependent decrease at the doses utilised, namely 5mg/kg, 10mg/kg and 20mg/kg. Thus the ability of a compound to reduce the degree of lipid peroxidation may indicate its value as a neuroprotectant.

Chapter 3

This chapter focusses specifically on the superoxide free radical and assessed the effect of various concentrations of DHEA on cyanide-induced superoxide anion generation. The ability of cyanide to cause a dose-dependent increase in superoxide anion generation was confirmed and it was

observed that DHEA isunable to reduce the concentration dependent induction of superoxide radicals by KCN at the concentrations used. Therefore, it is suggested that the neuroprotection provided by DHEA observed in other chapters is not mediated through the inhibition of the production of the superoxide anion, and another mechanism of action must exist.

Chapter 4

Apoptosis, a form of programmed cell death, was initiated with QA while rats received one daily subcutaneous injection of DHEA, at three different doses over two weeks. Hippocampal slices were stained using TUNEL and photomicrographs identified apoptotic cell death with a fluorescent marker. Apoptosis was inhibited at all doses, suggesting that DHEA is protective against QA and is preventing neurodegeneration, possibly by exerting an effect through enzyme modulation. The hippocampus is susceptible to neurodegeneration in AD and other pathological disorders related to aging, thus protection of hippocampal neurons by DHEA is relevant and provides an avenue for investigation of the clinical use of this neurosteroid in AD and other neurodegenerative diseases.

Chapter 5

Cresyl violet, a dye which stains Nissl substances purple, was used to identify and evaluate any morphological changes after the bilateral hippocampal injection of the neurotoxin QA in rats receiving daily subcutaneous injections of various doses of DHEA over two weeks. Photomicrographs showed that DHEA was able to inhibit cell death by preventing loss of cell integrity and maintaining morphological conditions at all doses utilised in the investigation. The results concurred with findings in the previous chapters.

Chapter 6

A potent free radical scavenger in the body is the hormone melatonin, the chief product of the pineal gland. This chapter investigated the influence of DHEA on pineal glands in culture to ascertain the effect, if any, on pineal indole metabolism. It was established that DHEA does not result in a significant change in the production of melatonin, but there is a significant decrease in

the concentration of serotonin/methoxytryptamine, prompting speculation that overall metabolism is increased without altering the relative amounts of each metabolite. A significant increase of N-acetylserotonin was observed, while yhe activity of HIOMT was not altered.

Chapter 7

Since the mechanism of action of the neuroprotective effects established in the preceding chapters is unknown and the importance of metal ions in neurodegeneration is well-researched, the potential interaction of DHEA with two metal ions, Cu(II) and Fe(III), was investigated using UV/VIS spectroscopy and electrochemistry. The spectroscopic study showed that the addition of both these metal ions results in a decreased absorbance and a shift to shorter wavelengths, indicative of a possible metal ligand interaction. This was further explored using electrochemistry, the results of which demonstrated that DHEA may have a weak interaction with these metals, but also highlighted the fact that more extensive study was required. The reduction in peak and shift to more negative potential in voltammograms indicates Fe(III) is becoming more difficult to reduce, which is a function of ligand interaction. However the results suggest that any interaction of this steroid with either Cu(II) or Fe(III) is weak.

General Conclusions

Thus, there is merit in the statement that DHEA has neuroprotective properties. The mechanism of this neuroprotection is unclear but the findings of this thesis suggest that it is not due to an inhibition of superoxide radical generation, nor is it due to an increased production of the anti-oxidant melatonin. DHEA was shown to preserve normal neuromorphology and reduce the incidence of apoptotic cell death in the presence of QA. There appears to be a weak nteraction between DHEA and the two metal ions under investigation, Fe(III) and Cu(II), and this may play an important role physiologically.

Further Studies

Although there has been much investigation into dehydroepiandrosterone, particularly over the last ten years, there are still many questions which are unanswered. The preceding chapters have highlighted some of these issues, which will be further discussed in the paragraphs below.

Observing the recovery of rats after intrahippocampal injection with quinolinic acid lead to the suggestion that a further behavioural study would be of value. Rats in the groups receiving DHEA experienced seizures which were relatively shorter and less severe than those experienced by the control group. A more extensive study, possibly evaluating the dose-dependence of this phenomenon would be of value in the future. After recovery from surgery, rats in the control group displayed the classic symptoms of an Alzheimic rat: stiff tail, glazed eyes, and a generally unkempt coat suggesting that the rat no longer cleans itself adequately. Rats also appeared more anti-social. Rats receiving all concentrations of DHEA did not display these characteristics to the same extent, once again suggesting that DHEA is reducing the amount of neural damage caused by QA. Further behavioural studies may be of value.

The literature regarding the effectiveness of DHEA or its sulphated ester in preventing seizures is, unfortunately, not conclusive. Budziszewska *et al*, (1998) demonstrated that DHEAS, along with other neurosteroids, has be shown to significantly increase the dose of NMDA required to produce clonic convulsions in 50% of tested animals (CD_{50}), while Hansen *et al* (2003) have demonstrated that the sulphated ester of DHEA is neither anticonvulsant nor antiepileptogenic. DHEA itself has not been well-documented, providing further evidence that further study would be beneficial.

Chapter 6 demonstrated the effect of DHEA in pineal glands in culture but further work should be carried out to determine the effect on pineal indole metabolism *in vivo*, since an *in vitro* model cannot completely replicate the brain environment. Although the incubation conditions have been optimised, the impact of other endogenous substances in the brain and the interaction with

enzymes has not been assessed. Also, the organ culture can not mimic physiological levels of DHEA, which differ throughout the brain, as mentioned previously.

Many researchers suggest that rodents are unsuitable for obtaining results which may be extrapolated to humans since these have minimal endogenous DHEA(S). These workers caution that these results should not be used to promote DHEA supplementation in human. Rather, these discoveries in rodent models can be used as targets for future research. There is also much debate over DHEA concentrations being tested in animals and humans and whether these doses should be pharmacological or physiological. This view may also be addressed in terms of the restoration of levels to those experienced in youth, or to raise circulating levels, irrespective of serum concentration.

Zhang *et al* (1999) demonstrated that DHEA and melatonin exert a synergistic effect on T-cell proliferation and Vitamin E levels in retrovirus infected and unaffected rats. The combined action of DHEA and melatonin warrants further investigation since both these hormones exhibit an age related decline and are affected by circadian rhythms. Both have been revealed to have a favourable effect on immune response, and both display anti-oxidant properties (Oreintreich *et al*, 1984; Southgate, 1998).

This thesis investigates the potential interaction between DHEA and two metal ions in Chapter 7, which revealed that the steroid may be interacting weakly with both these ions. A more comprehensive metal-ligand examination may prove fruitful in determining how DHEA is exerting its neuroprotective effects. During the electrochemical investigation supplementary scans with Cu(II) suggested that the interaction may be time dependent and further investigation is necessary. Thus, further knowledge of the potential time-dependent degradation would be beneficial. Additionally, light-dependent degradation could also be examined.

The interaction of DHEA with receptors – either an unidentified DHEA(S) specific receptor or a host of steroid specific receptors – must be better characterised and is integral to the understanding of the unestablished mechanism of action of DHEA.

DHEA, as previously mentioned, has been identified as a neurosteroid. This compound is thus synthesised both by the adrenal glands and the brain itself. Although various scientists have investigated the origin and fate of human DHEA and its sulphate derivative more understanding is necessary of the movement of DHEA in the body and the relationship between DHEA and DHEAS.

As noted in the literature review, DHEA is a pleiotropic hormone and the extent of the effects of DHEA in the human body in still unclear. More work must be directed at determining whether such effects are attributable to DHEA itself, levels of DHEAS as a circulating reservoir or of downstream metabolites such as progesterone, oestrogen and testosterone. The literature review also outlined the various effects of DHEA in the body currently identified and the potential therapeutic opportunities in manipulating serum levels must be explored, both peripherally as a steroid and centrally as a neurosteroid. Clinically, more research is necessary into the benefits of replacement therapy of DHEA in the elderly and in those with any form of pathophysiological deficiency. The therapeutic effects on well-being, mood or sexuality, irrespective of endogenous DHEA(S) levels must be further explored (Allolio & Arlt, 2003). To this end, a better characterisation of the pharmacokinetics of both DHEA and DHEAS is also necessary.

The age-related decline in DHEA is well documented (Oreintreich *et al*, 1984, Orentreich *et al*, 1992) and although work has been carried out to investigate the effects of lowered DHEA(S) levels in the elderly and the impact of raising these levels to the normal physiological range for those in the third decade of life, more attention must be played to the effects DHEA has during development and adolescence, when these levels are still increasing (Arlt *et al*, 1998; Arlt *et al*, 1999; Ceserini *et al*, 2000).

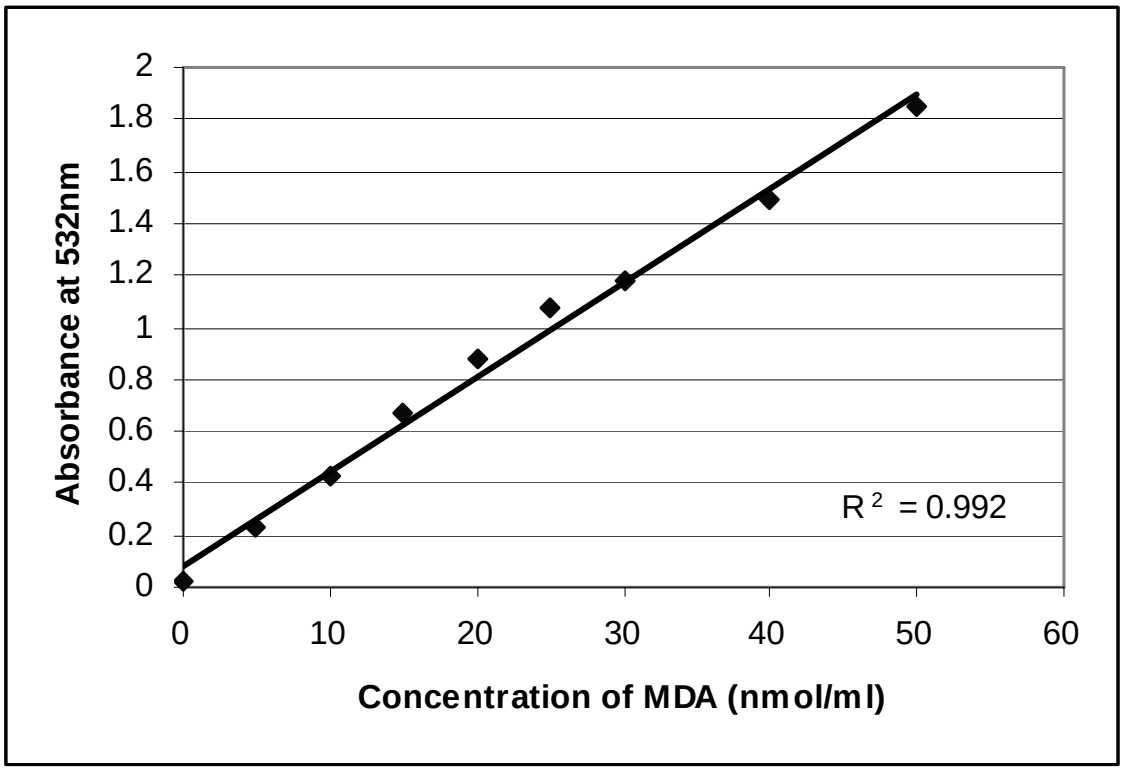
Wick (2002) is not alone in cautioning against the labeling of “anti-aging” supplements from a sociological standpoint. He argues that while many substances may or may not be efficacious, their irresponsible marketing may result in potential physical or economic harm, so responsible investigation of DHEA is necessary to dispel any myths surrounding its possible uses.

Appendix A: Animal Care

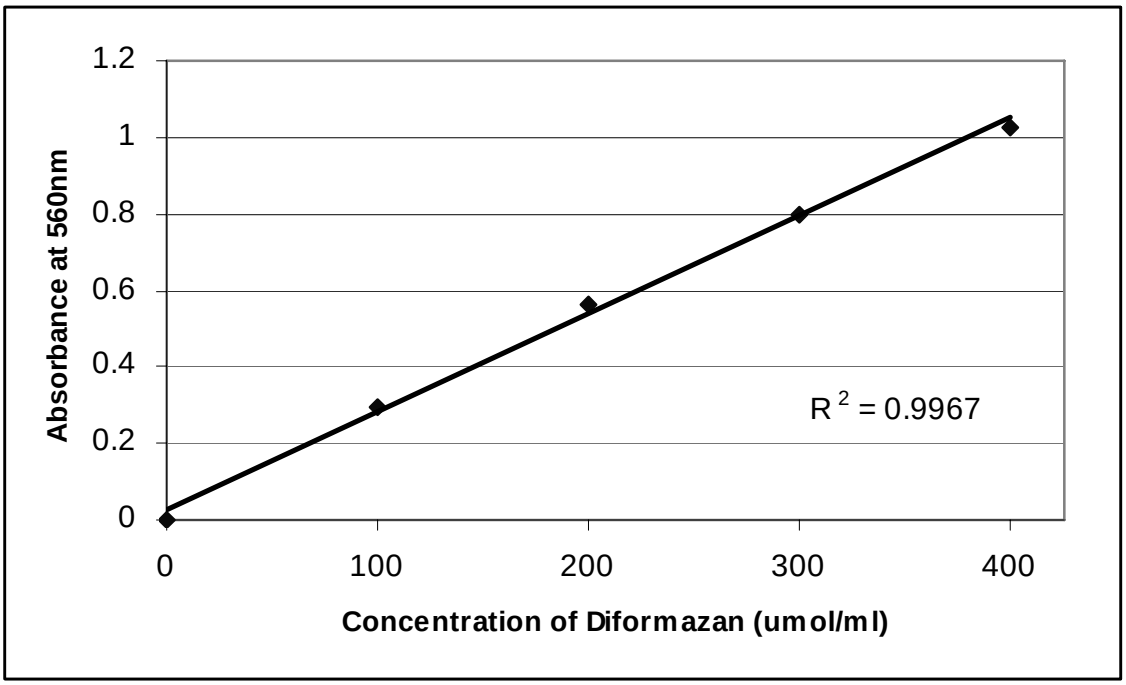
The animals used were adult male Wistar rats, purchased from the South African Institute for Medical Research, Johannesburg, South Africa. Animals were housed under artificial illumination (approximate intensity = 300 μWatts/cm^2) with a daily photoperiod of 12 hours with lights commencing at 06:00. The animal house temperature was maintained within the range 20 – 24° Celcius and an extractor fan ensured constant removal of stale air. The rats were housed at five per cage with food and water provided *ad libitum.* Cages were constructed of an opaque plastic, with metal grid floors and covers. Cages were cleaned regularly.

The protocol was in accordance with, and was approved by, the Rhodes University Ethics Committee.

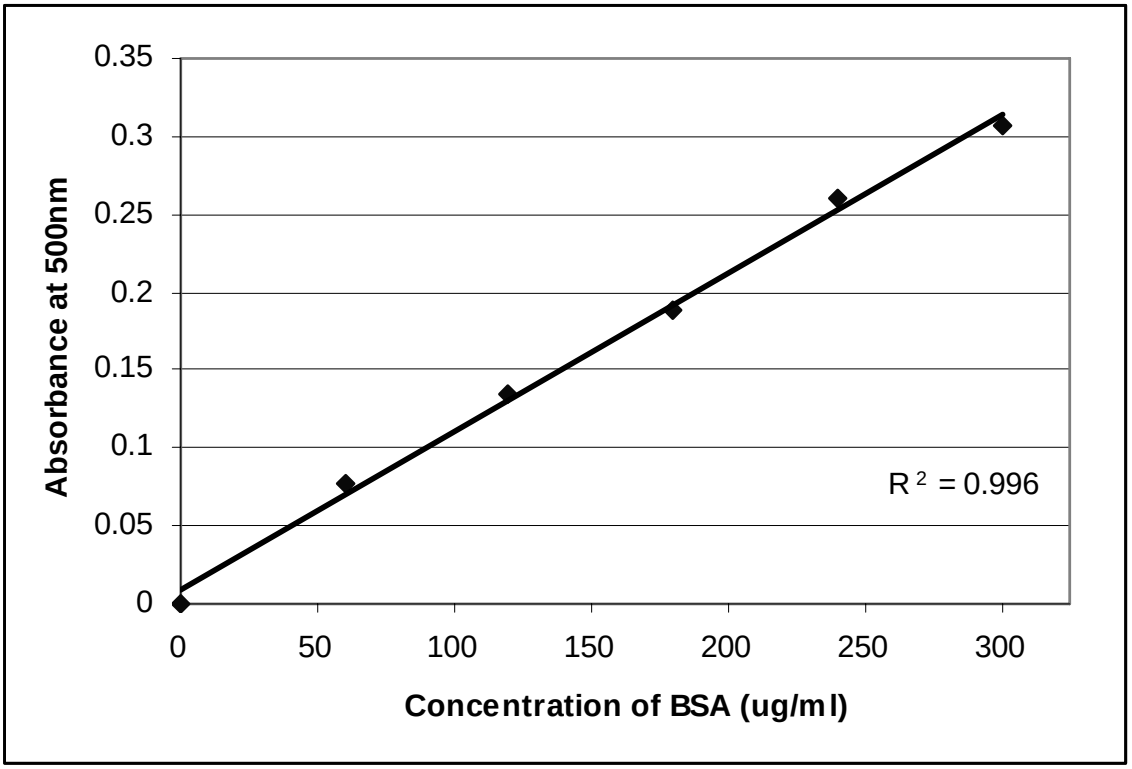
All efforts were made to minimise animal suffering and conserve animal resources.



The malondialdehyde calibration was obtained by preparing solutions of MDA over a range of concentrations and reading the absorbance of the thiobarbituric acid reactive species at 532nm as described in Section 2.2.1.3



The diformazan calibration curve was obtained by preparing solutions of NBD over a range of concentrations and reading the absorbance at 560nm as described in Section 3.2.1.3



The protein assay calibration curve was obtained as described in Section 3.2.1.4, according to a modified method of Lowry *et al* (1951).

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Image of effect of programmed cell death on organisms, Fig 1.10 from www.celldeath.de/aporeview

Image of hippocampus in the rat brain, Fig 1.3 from http://www.bio.davidson.edu/courses/genomics/method/Brainparts.html

Image of morphological changes visible during apoptosis and necrosis, Fig 1.9 from www.celldeath.de/aporeview

Image of phospholipid bilayer, Fig 2.1 from www.nature.com/horizon/livingfrontier/background/images/membrane_f2.jpg

Image of site of synthesis of DHEA, Fig 1.12 from www.med.unibs.it/~marchesi/sterhorm.html

Images of TUNEL test principle and labeling principle, Figs 4.1 and 4.3 from http://www.roche-applied-science.com/fst/apoptosis.htm?/sis/apoptosis/products/insitu_celldeath_detection.htm

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